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THE ROLE OF SMOOTH MUSCLE CELLS IN THE FIBROGENESIS OF ARTERIOSCLEROSIS

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While studying the structure and histochemistry of various types of arteriosclerotic lesions,[1-6] we have encountered a peculiar feature of the repair stage in some lesions. In the focal elevations representing either "insudation" of blood proteins into the intima or mural thrombi, the conversion of protein material to intercellular connective tissue elements frequently took place by means of an avascular form of organization.[6,7]

The purpose of this publication is to report investigations into the nature of the cells concerned with the avascular organization. In order to substantiate our impression that these elements were smooth muscle cells rather than fibroblasts, we have undertaken parallel morphologic and histochemical studies of the cells under discussion and also of known smooth muscle cells and fibroblasts.

MATERIAL AND METHODS

The material for this study consisted of human biopsy and necropsy tissue as well as biopsy tissue from experimental animals. Twenty-four blocks were selected from the following sources: 14 were removed at necropsy from the aorta and coronary arteries and represented arteriosclerotic lesions with the avascular type of organization; 5 blocks from human and 2 blocks from animal biopsy tissues were used for investigation of the classic type of granulation tissue containing fibroblasts; 2 blocks procured from human uterus and 1 block from human colon, both containing known smooth muscle cells, were utilized as controls.

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All the tissues were fixed in a modified formol-sublimate-acetic (FSA) solution† or Lillie's buffered 10 per cent formalin.[8]

Following the fixation time optimal for each fixative solution, the processes of dehydration, clearing, infiltration and embedding by either the conventional method using alcohol and toluol, or by means of tetrahydrofuran, were carried out as outlined elsewhere.[9]

Sections not exceeding 3μ in thickness were stained with hemalum-phloxine-saffron, Masson's trichrome, phosphotungstic-acid-hematein or phosphotungstic acid hematoxylin, Alcian blue-PAS-hematoxylin-orange G, Heidenhain's azan, pentachrome II, resorcin fuchsin-nuclear fast red-metanil yellow, and Bielschowsky-Maresch silver impregnation for reticular fibers-nuclear fast red-metanil yellow.

Results

A white or pearly white arteriosclerotic plaque often consisted of abundant, loose, intercellular acid mucopolysaccharide-rich ground substance, in which numerous cells were embedded. The longitudinal axis of these cells was almost always parallel to the surface of the endothelial lining so that such a plaque seemed to be composed of layers (Fig. 1). Each of the cells (Fig. 2) had a slender, tapering cytoplasm. The nucleus, which was usually situated in the widest portion of the cell body, was elongated, cigar-like in shape and had blunt ends. The chromatin granules of the nucleus were rather coarse. The nucleolus (at times 2), when visible, was small and not very conspicuous. It always showed the same staining quality as the remainder of the nucleus. The slender cytoplasm was not homogeneous, but embedded in its faintly eosinophilic homogeneous matter were numerous delicate and coarse, strongly eosinophilic fibrils. The coarser fibrils were situated at the cell boundaries, whereas the more delicate ones were seen in the cytoplasm proper. They were arranged longitudinally almost parallel to each other and to the longitudinal axis of the cell. The coarse fibrils extended beyond the cell boundaries, "communicating" with similar structures belonging to the neighboring cells, thus interlacing with each other. These fibrils were intensely red when stained with Masson's trichrome (Fig. 3) and gave a positive reaction with PTAH (Fig. 5).

On the other hand, known fibroblasts from areas of orthodox organization had an altogether different appearance and staining quality. The cytoplasm of a young fibroblast was slightly basophilic, and no eosinophilic fibrils associated with the cytoplasm were demonstrable with Masson's trichrome stain (Fig. 4). The cytoplasm was starlike in shape and had several elongated processes extending from its main body. The eccentrically situated nucleus was oval, flattened, and at times slightly indented. Its chromatin was very finely granular, giving the nucleus a

† FSA was prepared as follows: distilled water, 75 ml.; formaldehyde solution, 20 ml.; acetic acid, 5 ml.; trichloroacetic acid, 4 gm., and mercuric chloride, 4 gm.

"dusty" or foamy appearance. The nuclear membrane was distinct and accentuated. Similarly, the nucleolus (1 or 2) was very prominent and large. It had some affinity for acid dyes and was refractile. When stained with PTAH, the fibroblasts from human necropsy tissue showed no PTAH-positive fibrils (Fig. 6). It is known that fibroblasts do possess fibrils, the so-called Mallory's fibroglia,[10] which are associated with the cytoplasm. These are said to be present in fresh material and to be stainable with PTAH, but they disintegrate almost immediately post mortem and are thus not demonstrable in necropsy material. However, when fresh experimental animal tissue was fixed immediately following removal, PTAH-positive fibrils were demonstrable in association with fibroblasts (Fig. 7). It is not in the scope of this paper to debate whether the PTAH-positive fibrils seen in Figure 7 represent the connective tissue tonofibrils in the sense of German investigators,[11] or Mallory's fibroglia[12] and whether the tonofibrils and fibroglia may be considered to be identical. Of interest to us, however, is the fact that the distribution of these fibrils and their relation to the cell body, as well as their number and mode of extension into the surrounding tissue, was different from that of fibrils of the cells found in the avascular organization in arteriosclerosis (compare Fig. 5 with Fig. 7). On the other hand, the staining quality and the arrangement of the fibrils of the cells in the latter location were identical to the fibrils of known smooth muscle cells in our control sections from uterus and intestine. They were thus considered to represent myofibrils and will be referred to as such.

When the arteriosclerotic plaque was composed of very young connective tissue elements, the smooth muscle cells were often seen in a stage of apparent contraction. Thus, in a longitudinal section the cytoplasm was slightly broadened in some areas, and its poles with the extending myofibrils were "pulled" together. The nucleus exhibited a wrinkled, "snake-fence" appearance (Fig. 8), its length and shape being the expression of the degree of contraction of the cell.

Not only did these cells differ morphologically from fibroblasts, but they also differed in their relationship to the formed elements of connective tissue developing in the ground substance. The association of the smooth muscle cells with formed elements was a very intimate one. The first to develop in the area of avascular organization were delicate, abundant, silver-positive reticular fibers (Fig. 9), which were arranged in a parallel fashion and were most numerous adjacent to the cell and almost condensed to a shell around it. A higher magnification demonstrated the close association of these fibers with a smooth muscle cell. Some of the delicate silver-positive fibers seemed to be directly associated with the outer cell boundary (Fig. 10). In contrast, in the area of

conventional granulation tissue, similar fibers were developing primarily around the capillaries, and when present in the surrounding tissue, they were less delicate, less abundant and their relationship to the fibroblasts was less intimate (Fig. 11).

This also applied to the elastic fibers. In the area of avascular organization the elastic fibers were present very early in the process of repair and were most closely associated with smooth muscle cells (Fig. 12). However, in young granulation tissue containing capillaries and fibroblasts, the elastic fibers, if present, were very scanty and again not as closely associated with fibroblasts.

The relationship of PAS-positive material and collagen fibers to smooth muscle cells was similar. Thus, PAS-positive material was abundant in an early stage of avascular type of repair, surrounding the smooth muscle cells (Fig. 13). In orthodox granulation tissue it was seen mainly around the capillaries and when present in the surrounding tissue was arranged haphazardly and was not necessarily associated with the fibroblasts (Fig. 14). The last to appear were collagen fibers; however, the pattern of their development followed that of the other extracellularly formed connective tissue elements closely in that they were seen surrounding the smooth muscle cells in an almost regular, parallel arrangement (Fig. 15). In the young conventional type of granulation tissue, however, the collagen bundles were coarser, developed in a criss-cross fashion, and were not closely related to the boundaries of the fibroblasts (Fig. 16).

Very early in the process of repair, young smooth muscle cells were seen expanding in the acid mucopolysaccharide-rich metachromatic ground substance with little, if any, formed connective tissue elements surrounding them (Fig. 8). Because the myofibrils extended from the cytoplasm into surrounding tissue, the two polar boundaries of the cells were ill-defined (Figs. 2, 3 and 5). As the various types of connective tissue fibers developed around smooth muscle cells, a limitation was imposed on the expansion of the myofibrils beyond the cell boundaries, and the cell thus became confined to a well delineated space (Fig. 17). As the connective tissue fibers matured and aged and particularly when the cell-enclosing collagen fibers became denser, the contractile elements of the smooth muscle cell—the myofibrils—were no longer demonstrable. The first intracellular elements to atrophy (and disappear) under these circumstances were the delicate myofibrils, while the coarse ones at the outskirts of the cytoplasm were still preserved (Fig. 17). Later in the process of sclerosis of the plaque, the coarse myofibrils, as well as the nucleus and cytoplasm, atrophied, leaving an almost empty, clear, elongated space outlined by dense collagen. At times a nuclear "shadow" was still present in this clear space (Fig. 18).

Discussion

The peculiar type of avascular organization encountered in arteriosclerosis has been previously described by Rössle.[13] He observed that the "insuded" blood proteins were converted into the sclerotic plaque by means of an avascular type of organization. Rössle remarked that such an area was poor in fibroblasts, but he did not mention the presence of other cells. The same type of organization was noticed by British workers[14] in regard to the incorporation of mural thrombi. According to them, the cells concerned with this type of organization were fibroblasts.

Present studies confirm our earlier observations[4,6,7] that we are, indeed, dealing with smooth muscle cells rather than with fibroblasts as postulated by others. Morphologically and histochemically these cells are identical to known smooth muscle cells and are entirely different from known fibroblasts. Thus, the shape of the cell and the nucleus with its coarse chromatin distribution (Fig. 2); the eosinophilia of the cytoplasm (Figs. 2 and 3); the presence of numerous fibrils which are parallel to the longitudinal axis (Fig. 2) and extend out of the cytoplasm into the connective tissue (Figs. 3 and 5); the intense eosinophilia of these fibrils (Fig. 3) and their affinity for PTAH (Fig. 5); the contractility of the cell which lends the characteristic "snake-fence" appearance to the nucleus (Fig. 8); and the close association of the cell with elastic fibers coating it (Fig. 12), are all features identical to those of the control smooth muscle cells in the uterus and intestine. On the other hand, the corresponding features were altogether different from those of fibroblasts (Figs. 4, 6, 7 and 11).

Since the cells found in avascular granulation tissue are smooth muscle cells, it follows that these are capable of elaborating intercellular connective tissue components. The association of the cells with all forms of extracellular connective tissue elements was, indeed, very intimate (Figs. 9, 10, 12, 13 and 15); this association has also been observed by others[10,15–17] prior to our investigation. In regard to developing smooth muscle cells, Maximow and Bloom stated: "The reticular fibers between the muscle cells are probably produced by the same cells which become muscle fibers—the developing smooth muscle cells function as both myoblasts and fibroblasts."[10] D'Antona[16,17] considered smooth muscle cells to possess a pronounced fibrogenic power.

The origin of smooth muscle cells in areas of avascular granulation tissue in arteriosclerosis is uncertain. This applies also to other unusual sites, as in organizing thrombi other than in arteriosclerosis[18]; in the intima of either experimentally injured[19] or ligated[20] arteries, and in some arteriosclerotic (experimental and human) lesions[21,22] ex-

cept those in the avascular type of organization of blood proteins. Under certain circumstances smooth muscle cells are said: to develop from "transitions" of fibroblasts [15,23,24]; to represent transformed endothelial cells [20,25–30]; or to migrate from the media into the intimal arteriosclerotic lesions through naturally occurring gaps in the internal elastic lamina.[18–20] It would appear from our observations that in the areas of avascular organization the smooth muscle cells are derived from endothelium. In the process of organization of mural thrombi, the endothelial cells multiply and stretch to cover the thrombus.[5,14,31] They then migrate into the substance of the thrombus [14,29,31] and may differentiate into smooth muscle cells. During the process of insudation of blood proteins into the intima, the endothelial cells may be injured. Regeneration follows, and it is conceivable that in the course of multiplication and migration into the intima, differentiation occurs, giving rise to a new cell type, the smooth muscle cell.

Summary and Conclusions

The cellular elements involved in the repair phase of many arteriosclerotic lesions (i.e., organization of insudate and mural fibrin deposition) were compared with known fibroblasts and smooth muscle cells. These cells possessed all the morphologic features characteristic of smooth muscle cells.

Their ingrowth into the affected intima seems to meet the pathophysiologic requirements for an element not only capable of organizing the blood protein deposit and thus elaborating connective tissue elements, but also rendering the area "elastic" by virtue of its contractility.

It would appear from our observations that the smooth muscle cells in the above-mentioned situations are derived from endothelium, which regenerates following damage in the course of the arteriosclerotic process.

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LEGENDS FOR FIGURES

FIG. 1. An eccentric pearly-white arteriosclerotic plaque in a human coronary artery contains abundant acid mucopolysaccharide-rich ground substance (blue in section, light gray in photograph). Embedded in it are numerous elongated cells with longitudinal axes parallel to the endothelial lining. Alcian blue-periodic acid-Schiff (PAS)-hematoxylin-orange G stain. × 244.

FIG. 2. Smooth muscle cell. The cell has a slender, tapering, slightly eosinophilic cytoplasm (pink in section, dark gray in photograph). The nucleus is elongated, cigar-like in shape, with blunt ends. Coarse chromatin granules are distributed uniformly throughout the nucleus. A small nucleolus is inconspicuous. Hemalum-phloxine-saffron stain. × 972.

FIG. 3. Extending from the cytoplasm of a smooth muscle cell are intensely eosinophilic myofibrils (black in photograph, deep red in section), interlacing with similar myofibrils of neighboring cells. Masson's trichrome stain. × 972.

FIG. 4. Young fibroblast in granulation tissue. The nucleus is oval. Its chromatin granules are very fine and 2 nucleoli are prominent. The cytoplasm is stellate in shape and basophilic (dark gray in photograph, bluish in section). Eosinophilic fibrils are not present. Masson's trichrome stain. × 1460.

FIG. 5. Smooth muscle cell. The myofibrils which stained red with the Masson trichrome stain (Fig. 3) are positive with phosphotungstic acid-hematoxylin (PTAH; black in photograph, dark blue in section). PTAH stain. × 1460.

FIG. 6. Young fibroblast in granulation tissue (human necropsy tissue) does not possess PTAH-positive fibrils. Stain and magnification as in Figure 5.

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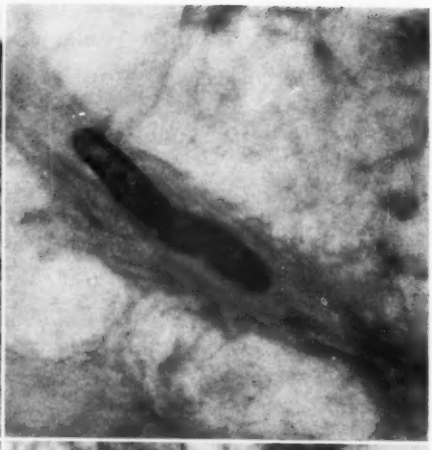

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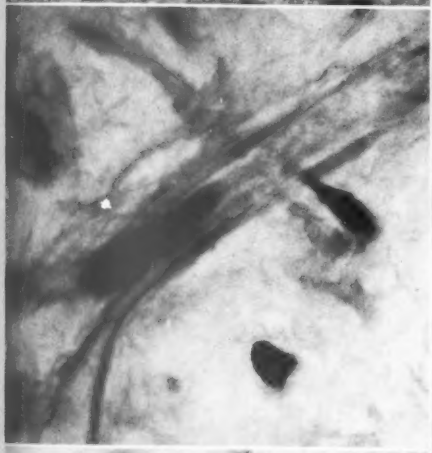

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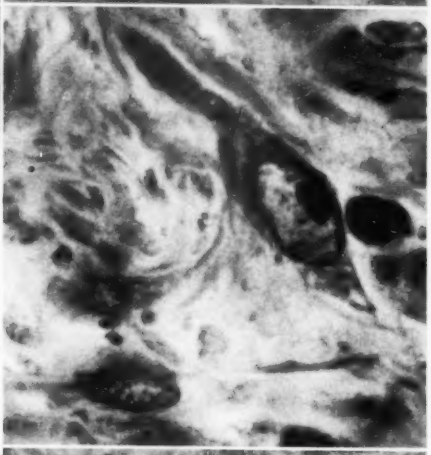

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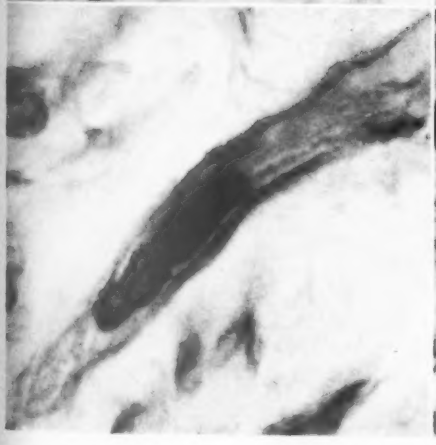

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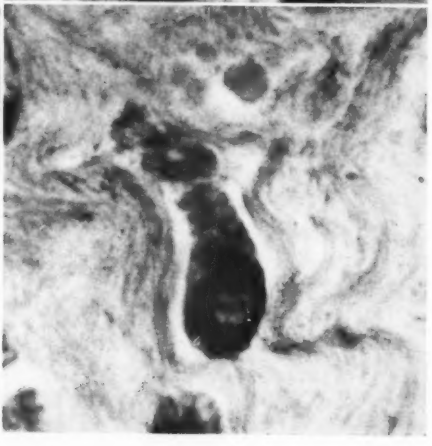

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FIG. 7. Young fibroblast in granulation tissue from fresh experimental animal tissue shows PTAH-positive, delicate fibroglia (black in photograph, dark blue in section). Note the difference in number and arrangement between the fibroglia and myofibrils seen in Figure 5. PTAH stain. × 972.

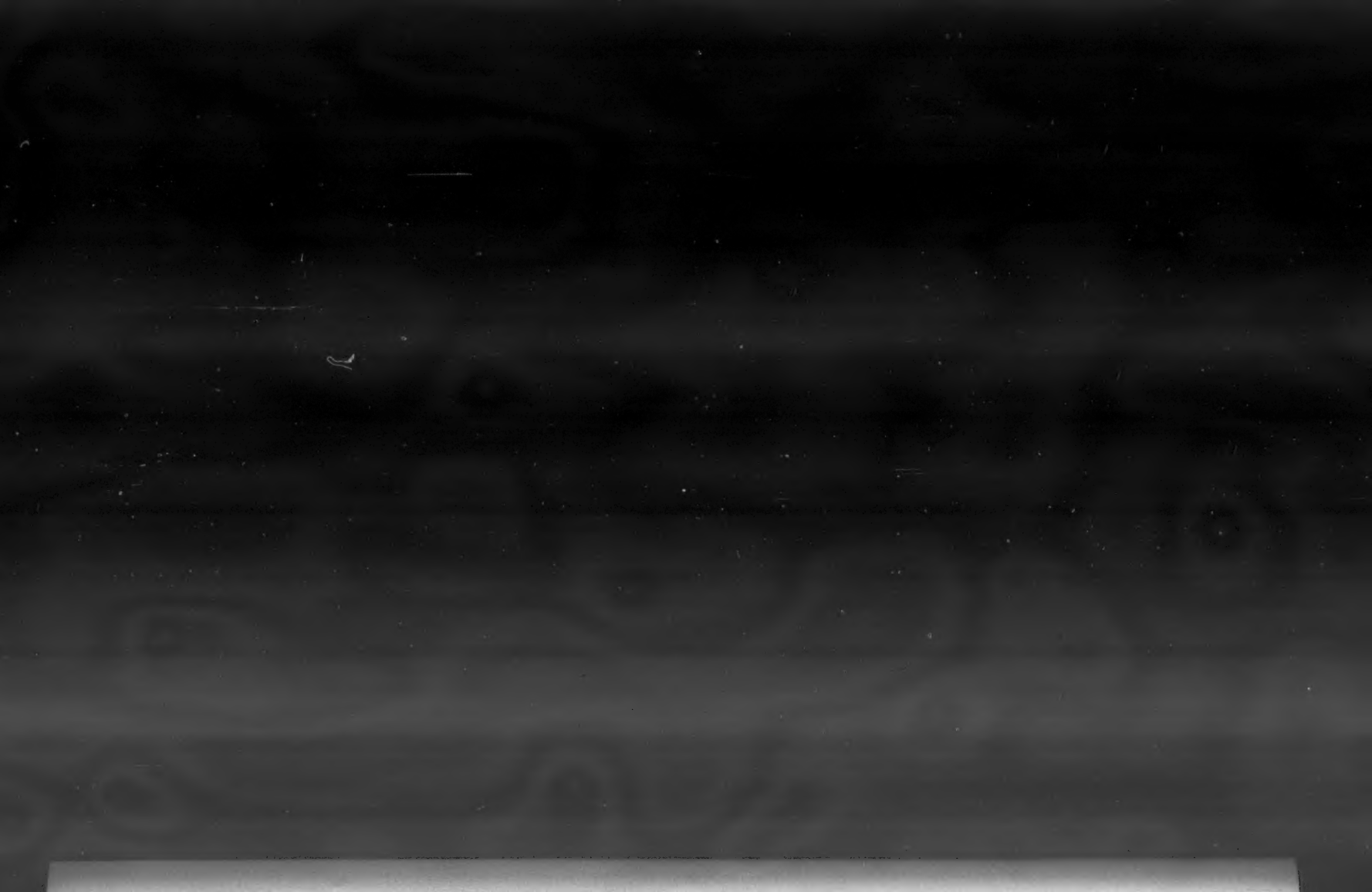
FIG. 8. Very young, smooth muscle cell in a juicy, abundant ground substance; formed connective tissue elements are not yet present. The cell is in a stage of contraction, represented by a snake-fence appearance of the nucleus and broadening of the central portion of the cytoplasm. Note the "pulling" exerted upon the myofibrils at the upper pole of the cytoplasmic body. Heidenhain's azan stain. × 972.

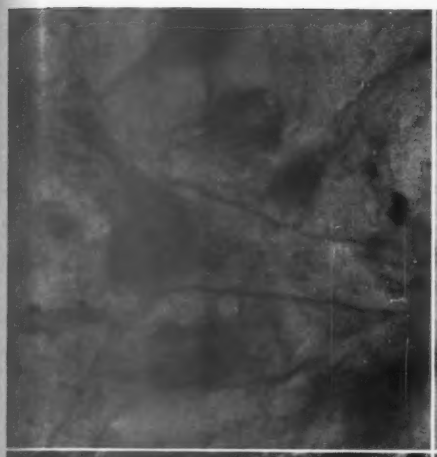
FIG. 9. An area of avascular organization in an arteriosclerotic plaque. Innumerable delicate, wavy, silver-positive reticular fibers are arranged in an almost parallel fashion and surround smooth muscle cells (lower right). Bielschowsky-Maresch silver impregnation for reticular fibers-nuclear fast red-metanil yellow stains. × 244.

FIG. 10. Higher magnification of the right lower corner of Figure 9. The black, silver-positive reticular fibers are intimately associated with the smooth muscle cell. Very delicate fibers are closely applied to the cell's boundaries. Stain as in Figure 9. × 972.

FIG. 11. Human granulation tissue. The silver-positive, black reticular fibers are best developed around the capillary (center). They are coarser than those seen in Figure 9, and their arrangement is not parallel but a criss-cross one. Stain and magnification as in Figure 9.

FIG. 12. Elastic fibers are closely applied to the boundaries of the smooth muscle cells in an avascular organization. Resorcin fuchsin-nuclear fast red-metanil yellow stain. × 972.





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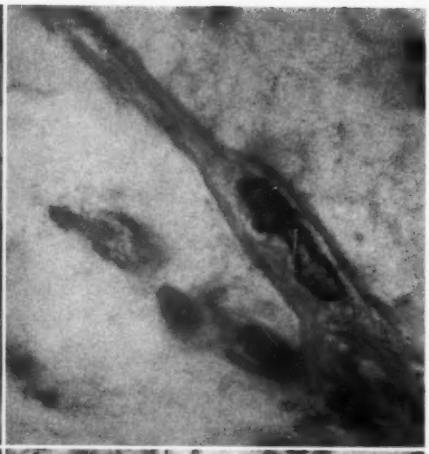

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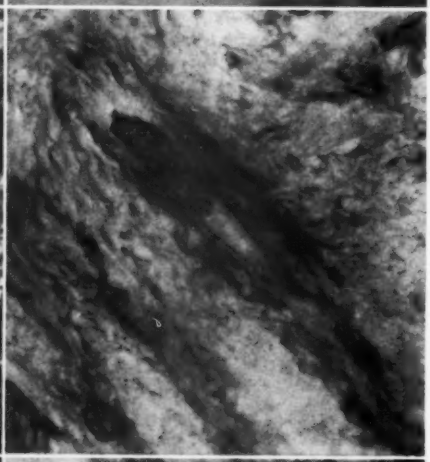

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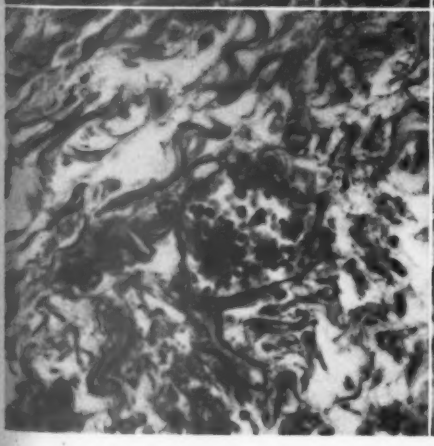

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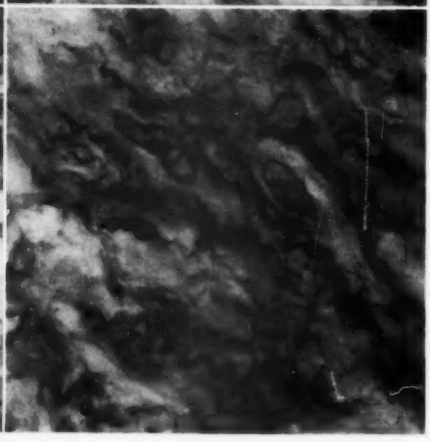

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FIG. 13. Embedded in loose, young, acid mucopolysaccharide-rich ground substance (gray in photograph, blue in section) are smooth muscle cells surrounded by PAS-positive material (black in photograph, purplish-red in section). Alcian blue-PAS-hematoxylin-orange G stains. × 972.

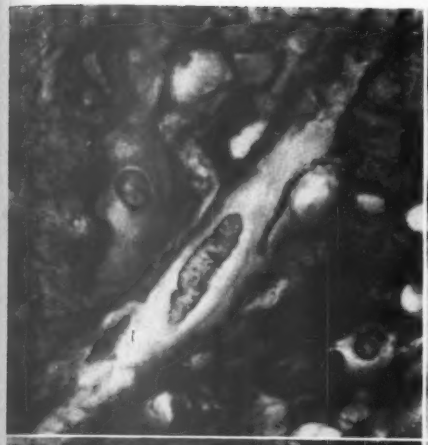
FIG. 14. In young conventional granulation tissue the PAS-positive material is outlining a capillary at the left upper corner (dark gray in photograph, purplish-red in section). Slightly PAS-positive substance is also developing in the surrounding tissue (gray in photograph, slightly purplish-red in section), but no close association can be seen between this and the fibroblasts. Stain as in Figure 13. × 1460.

FIG. 15. Avascular organization. Collagen fibers (dark gray in photograph, red in section) develop in a parallel fashion around smooth muscle cells in the ground substance (light gray in photograph, blue in section). Pentachrome II stain. × 244.

FIG. 16. Young granulation tissue. The coarse collagen fibers (dark gray in photograph, red in section) are developing in a haphazard fashion. Compare with Figure 15. Stain and magnification as in Figure 15.

FIG. 17. A smooth muscle cell is seen enclosed by dense collagen fibers, especially at its upper half (black in photograph, dark red in section). The cell is thus confined to a space and myofibrils no longer extend from the cell's cytoplasm into surrounding tissue. No delicate myofibrils are seen. Remnants of the coarse myofibrils are situated at the left, between the nucleus and cell's border. Pentachrome II stain. × 1460.

FIG. 18. Hyalinizing arteriosclerotic plaque. Dense collagen bundles (dark blue in section, black in photograph) outline elongated clear spaces previously occupied by smooth muscle cells. Nuclear "shadows" are seen in some of these spaces. Heidenhain's azan stain. × 972.



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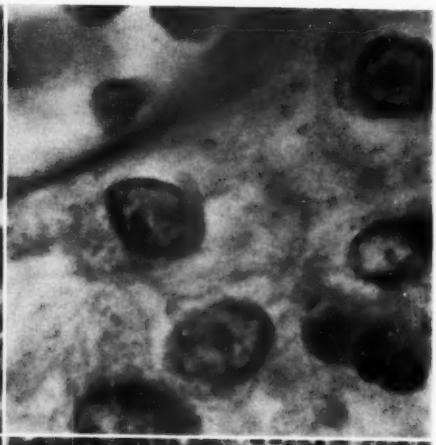

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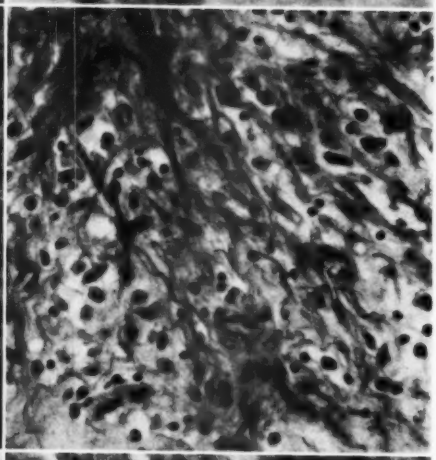

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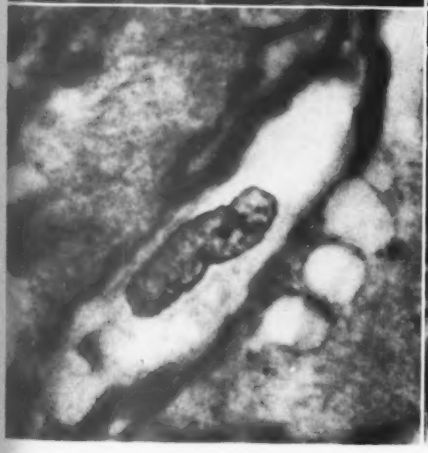

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CHRONIC PULMONARY EMPHYSEMA
(An Experimental Study)

III. Experimental Pulmonary Emphysema

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In the past, many different methods of producing pulmonary emphysema experimentally have been employed.

Bayer,[1] in 1870, in an unstated number of rabbits, reported emphysema at the margins of the contralateral lung after periods of half an hour or more of pneumothorax. Kläsi,[2] in 1886, saw marginal emphysema in one rabbit after 9 days. The descriptions indicate that they were dealing with chronic marginal emphysema of the spontaneous variety.

Riegel and Edinger,[3] in 1882, and Sihle,[4] in 1903, using vagal stimulation in dogs and rabbits, produced simple overdistention of the lungs. However, Brown-Séquard,[5] in 1885, stated that even brief vagal stimulation produced emphysema in rabbits.

In 1900, Bullara,[6] in dogs, and Cousteau,[7] in rabbits, claimed, without employing controls, to have produced emphysema by nasal obstruction for periods of 2 weeks to 7 months. Köhler,[8] in 1878, narrowed the trachea by lead wire and reported vesicular and interstitial emphysema in about 20 rabbits after 3 to 4 weeks. Neither controls nor diagnostic criteria were mentioned. In the same year, Hirtz[9] narrowed the trachea by ligature and claimed the production of generalized and marginal emphysema in 2 rabbits after 4 and 9 days. Sudsuki,[10] in 1899, ligated the trachea of 9 rabbits. In 3, surviving between 46 and 84 days, tracheal stenosis was found at necropsy. One showed marked marginal emphysema. In 1925, Nissen and Cokkalis[11] inserted a metal ring into the tracheas of 5 cats and found histologic vesicular emphysema in 3. Nissen[12] later obstructed the tracheas of 3 dogs, 4 rabbits, and 3 cats by packing the mediastinum with wax or plaster of Paris. After 3 to 6 months, local traces of true emphysema were found

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in 5, and 3 showed more marked lesions. Nissen employed controls in neither study. Loeb,[13] in 1930, kept 8 of 24 dogs alive for up to 14 months after the insertion of a narrow brass tube into the trachea. He found doubtful dilatation of alveoli but no emphysema.

In 1909, Priese[14] and Schall[15] employed face masks in 7 rabbits and 3 dogs. Daily application for between 3 and 11 months produced no emphysema. In 1917, Friedman and Jackson[16] in three 8-hour experiments, obstructed expiration by an intratracheal T-tube and valve and without giving details, stated that emphysema was present. Similar claims were made by Pfanner,[17] in 1922, in acute experiments with rabbits and dogs. Harris and Chillingworth,[18] in 1919, obstructed expiration by an intratracheal ball valve in 25 dogs, all of which survived 2 to 21 days and were reported to show some degree of emphysema. Their illustrations do not suggest more than a marked degree of distention of the lungs, and Loeb[13] refused to accept these changes as emphysematous. These investigators used an unstated number of controls with "nonfunctional" valves.

Kountz, Alexander and Dowell[19] in 1929, used the method of Harris and Chillingworth in 16 dogs and concluded that emphysema had been produced on the basis of raised intrapleural pressure. No anatomic details were given. In 1945, Sciuto[20] used the same method in dogs. His illustrations are not convincing. Hinshaw,[21] in 1938, used an improved ball valve in various animals for up to 18 months and observed frequent subpleural emphysematous vesicles. In 1940, Paine[22] devised an intratracheal flap valve. Three controls, with nonfunctional valves, showed minimal histologic emphysema after 9 weeks. Of 10 dogs which survived from 5 to 23 weeks with expiratory obstruction, 1 was normal, 3 had minimal alterations while 6 had definite acceptable microscopic emphysema. Of 9 dogs which survived from 4 to 30 weeks with inspiratory obstruction, definite macroscopic and microscopic emphysema was present in 6. Paine assessed his material critically, and his illustrations show convincing chronic emphysema.

In 1927, Nissen[12] enlarged the thorax in 18 dogs by various means. Ten survived for periods of between 2 weeks and 5 months. No emphysema was seen. Paine,[22] in 1940, also enlarged the thorax in 9 dogs by suturing "reefs" in the diaphragm. Two died. Seven survived for periods of 16 to 25 weeks. All but one had naked-eye emphysema, and in all 7, significant microscopic emphysema was present. Paine considered this method superior to valvular obstruction.

Most of the papers concerned with relative increase in the size of the thorax are studies of the fate of the remaining lung after pneumonectomy, rather than deliberate experimental studies of emphysema. Möll-

gaard,[23] in 1909, performed pneumonectomy on three 7-day-old pups which survived for 6 to 14 weeks, and 5 adult cats which survived for 14 days. The remaining lungs were enlarged, but he regarded this as hypertrophy. But his Figure 7 is more convincingly indicative of emphysema than many others in the literature.

Nissen,[12] in 1927, produced convincing chronic compensatory emphysema in cats and dogs after periods of up to 16 weeks following ligation of selected main bronchi and branches of the pulmonary arteries. Adams and Livingstone,[24] in 1932, claimed that the remaining lung tissue in 28 dogs, 2 to 12 months following lobectomy or pneumonectomy, showed various degrees of compensatory emphysema. They gave no details, and their histologic illustration is not convincing. Rienhoff, Reichert, and Heuer,[25] in 1935, in a similar but more detailed and controlled study of 10 dogs for periods of up to 6 months after pneumonectomy, found no emphysema in the remaining lungs. Their Figure 4 is more suggestive of emphysema than many for which positive claims have been made. Kountz, Alexander and Prinzmetal,[26] in 1936, removed up to 80 per cent of the lungs in 19 dogs and implied that emphysema developed in the remaining lung tissue but gave no necropsy details. Longacre and Johansmann,[27] in 1940, compared the long-term effects of pneumonectomy in 2 adult dogs and 3 puppies. After 2 to 4 years no emphysema was found in the "puppies," but they reported true chronic emphysema in both the "adults." However, only their Figure 11A is suggestive of emphysema.

In 1927, Campbell[28] exposed an unstated number of animals, including rabbits and mice, to lowered oxygen tensions for prolonged periods. He noted, incidentally, that "portions of the lungs of most animals were emphysematous." Prinzmetal,[29] in 1934, in a deliberate attempt to produce emphysema, exposed an unstated number of rats to only 8 per cent oxygen for 10 weeks. His illustration is convincing, but no details were given and no controls were employed.

In 1913, Caradonna[30] studied the effect of increased respiratory effort on the alveolar pores of an unstated number of young guinea pigs. One group was kept undisturbed as controls. Two other groups were whipped 4 times daily until they were in a state of collapse. Only occasional pores were seen in the controls between the ages of 5 and 15 months. In the whipped animals, pores became visible at 3 months and increased in number until, at 1 year, the lungs were frankly emphysematous. Kelman,[31] in 1919, used rabbits. Seven were exposed to intermittent tracheal inflation, 3 were killed by anaphylactic shock, and 18 were inoculated intratracheally by *Hemophilis influenzae* cultures or culture filtrates. She claimed that marginal emphysema was present in all these animals.

No precise criteria were given, and no controls were employed. Rasmussen and Adams,[32] in 1942, overinflated the lungs of 7 dogs for periods of 15 minutes twice weekly. The dogs survived from 1 week to 11 months. Vesicular emphysema was noted in one animal only. They did not claim that this was the result of the experimental procedure.

The literature suggests that many claims to the production of emphysema experimentally are unacceptable because of lack of controls, failure to state precise criteria for the diagnosis of emphysema, or the failure of illustrations to substantiate claims made in the text. The most careful study was that of Paine[22] in 1940. He assessed his results very critically and supplemented his anatomic studies by measurements of the intrapleural pressure by the method of Christie and McIntosh.[33]

Quite apart from the validity of the experimental work, nearly all the methods have been designed to produce abnormal distention of all or part of the lungs or to increase the amount of functional stress placed upon the lungs. The claims of the experimental workers lend support to the mechanical theories of pathogenesis of emphysema and show that different remote mechanisms may alter the conditions of respiration and produce emphysema. The methods employed do not shed any light on the intimate mechanism of pathogenesis and, indeed, few of the authors have commented on this aspect of the problem. Paine[22] regarded the development of emphysema as the result of physical stress on the alveolar walls. In passing, he mentioned the possibility of nutritive disturbances, due to capillary occlusion in the course of distention.

The Present Study

The present study resulted from observations on human lungs in thick sections in which it is obvious that the capillary bed is a major constituent of the alveolar wall. It seemed possible that the appearances of chronic emphysema might be produced if the capillary bed could be destroyed. Study of the literature[34] showed that there is ample evidence for regarding chronic emphysema as an atrophy of lung tissue. Further, it has been suggested that ischemia is at least an ancillary factor in the production of emphysematous atrophy.

If chronic emphysema can be regarded as an ischemic atrophy, it should be possible to produce emphysema by interfering with the blood flow through the pulmonary capillaries without the presence of mechanical overdistention of the lungs.

To test this hypothesis, repeated intravenous injections of a particulate substance were used in an attempt to cause enough vascular obstruction to produce tissue ischemia.

Material and Methods

Histologic Technique

The methods described in the previous section[35] were again employed. However, at least one block was taken from each of the major lobes of each pair of lungs. Additional blocks were taken as necessary. Thick sections cut at 100 μ were used routinely for the study of the emphysematous lesions.

Experimental Method

The method thought most likely to produce an adequate degree of vascular obstruction was the oft-repeated intravenous injection of a particulate substance of such a size as to produce blockage of the capillaries and precapillaries. It was necessary that the substance be nontoxic systemically, nonirritant locally and insoluble in body fluids.

Caledon blue R.C. seemed a likely substance. This is an anthraquinone dyestuff, 3′,3′ dichloro-indanthrone, which is insoluble in water and organic solvents. It was possible to obtain samples which had a majority particle size between 10 and 25 μ. The dye was supplied as an aqueous paste containing 10 to 12 per cent total solids as Caledon blue. Trials showed that a suitable dilution for intravenous use was an approximate 3 per cent of total solids. The diluent was 0.85 per cent saline with 0.05 per cent of a dispersing agent, Dispersol T, added.

Preliminary trials in mice showed that the material was nontoxic. Tissue reaction was minimal. It became obvious that, due to aggregation of the particles, it would not be possible to block at will vessels of a definite caliber by exact selection of particle size. This was no disadvantage, for it was not possible to get batches of Caledon blue of exactly the same particle size. In rabbits, as in mice, there was no immediate tissue reaction to the Caledon blue, but after a day or two there was a slight histiocytic response at the site of lodgment. Even this might be absent. Otherwise Caledon blue produced no tissue response. This was true even in rabbits which received repeated injections of the dye for periods of a year or more. At no stage was any granulomatous reaction or fibrosis produced.

In rabbits, the majority of the particles lodged in the precapillaries and to a lesser extent in the capillaries themselves. Even after a single injection, the blockage was the result of aggregates of particles rather than of individual particles. With repeated injections the aggregation became more marked, with the result that the vessel became grossly dilated around the large mass of dye. After prolonged injections, aggregates occurred in terminal arterioles but not larger vessels. The histologic appearances suggested a very marked degree of vascular obstruction in the lungs, but neither thrombosis nor infarction were produced. Not all of the particles were retained in the lungs, and Caledon blue became lodged in the vessels and taken up by the reticuloendothelial tissue of other organs.

Details of Experiments

First Experimental Series (22 rabbits). A suitable initial dose was found to be 2.5 ml. of the diluted Caledon blue, containing approximately 3 per cent of Caledon blue.

This was injected slowly into an ear vein over a period of 1 to 2 minutes. On a first injection, about 1 in 5 rabbits died following tachypnea and convulsions. In about half the rabbits, tachypnea commenced during or immediately after the injection but lasted only 2 to 3 minutes, after which the animals remained well. After 2 or 3 injections at weekly intervals, the dose was increased to 3 ml., and this was maintained until the end of the experiments. After the rabbits had been injected weekly for about 2 months, it was realized that aging might interfere with the assessment of the results. Consequently, further rabbits added to the series were selected

as appearing to be less than one year old. The weekly injections were continued until the survivors had been receiving them for over a year. The survivors were sacrificed by stunning, one to two weeks after their last injection.

During the collection of a control series, it became evident that spontaneous emphysema developed in rabbits. Details of this have been given in the preceding section.[85] The most important fact to emerge was that some degree of generalized emphysema developed in just over 50 per cent of rabbits over the age of 2½ years.

While it was thought that over half of the animals in the first experimental series were under two years old at the end of the experiment, it was impossible to be sure of this, and it was felt that the controlling was inadequate.

Second Experimental Series (25 "pairs" of rabbits). The experiment was repeated using rabbits of known ages. Most of these were 8 or 9 months old at the start, the extreme ages being 7 and 12 months. The controls were the paired litter mates of the animals which received the Caledon blue. Encouraged by the results in one of the animals in the first series which had received injections thrice weekly, the rabbits in the second series received injections twice weekly after the second week. The dosage was maintained at 2.5 ml. of diluted Caledon blue (approximately 3 per cent total solids) since there was a higher death rate in the second series as compared with the first. The injections were continued twice weekly until the survivors had received Caledon blue for 24 weeks. These were sacrificed by stunning 1 to 2 weeks after the last injection so that no rabbit was more than 18 months old at the conclusion of the experiment.

The litter mate controls were treated identically, so far as possible, to the experimental animals. Each received a biweekly injection of 2.5 ml. of 0.85 per cent saline with 0.05 of the dispersing agent added, but without the Caledon blue, at the same time the experimental animals were injected. The controls were sacrificed by stunning when their experimental litter mates died or were killed. The control lungs were processed in strict parallel to the experimental lungs at all stages.

In this series 25 pairs of rabbits survived for between one and 24 weeks. (One of the "pairs" consisted of 3 litter mates, of which 2 were given Caledon blue and the third retained as a control.)

Assessments of Results

In view of the experience of spontaneous emphysema in rabbits described in the previous section,[85] the results were assessed solely upon a comparison of the incidence of microscopic generalized emphysema in the injected animals and the controls.

Emphysema in marginal lobules was ignored, as the lobules are of inflammatory origin. Vesiculation is usually a marginal accentuation of generalized emphysema and did occur more frequently in the injected animals. As some cases of vesiculation may be the result of local inflammatory changes, it was ignored in the assessment. Vesiculation was, however, a very useful naked-eye guide as to the success of any individual experiment (Fig. 1).

Microscopic Appearances. The generalized and marginal emphysema found in rabbits injected with Caledon blue was identical in every respect to spontaneous rabbit emphysema[85] and to the classical descriptions of chronic emphysema in humans[84] (Fig. 2). The lesion was complicated, however, by the presence of masses of Caledon blue in the vessels. This did not modify the histologic appearance of the emphysema. There was destruction of the alveolar walls by fenestration (Fig. 3), which, at the margins, progressed until there was complete destruction and disappearance of the alveolar septums and fusion of neighboring alveolar sacs and ducts (Fig. 4). The changes in the elastic fibers were the same as in spontaneous emphysema.

Assessment of the Degree of Generalized Emphysema. An arbitrary system of plus grading was adopted. This was based on the extent and severity of the destruction of lung tissue by fenestration. Alveolar pores occur in all rabbit lungs and, as the emphysematous process consists of the development of abnormal numbers of fenes-

TABLE I

DETAILS OF FIRST EXPERIMENTAL SERIES

Rabbit no.	Body wt. at death (kg.)	Duration of Caledon blue injections (wk.)	Total Caledon blue *	Macroscopic			Microscopic				Degree of generalized interstitial pneumonia
								Degree & type of emphysema			
				Normal	Marginal lobule	Vesiculation	Nonemphysematous	Marginal lobule	Vesiculation	Generalized	
R. St./											
13	2.41	1	5.5	+	−	−	+	o	o	o	+++
4	2.41	3	8.5	+	−	−	+	o	o	o	+
6	3.95	5-1/7	15.0	+	−	−	−	o	o	+	+++
9	2.13	5-1/7	15.0	+	−	−	+	o	o	o	+++
17	2.23	6-5/7	17.5	−	−	+	−	o	+++	o	o
20	2.64	11-5/7	30.0	−	+	+	−	+++	+++	o	+++
26	2.43	14-2/7	39.0	−	+	+	−	+	++	+	o
24	2.03	15-5/7	42.0	−	−	+	−	o	++	++	+++
16	2.33	17-6/7	48.0	−	−	+	−	o	+	+	+
28 †	2.34	31-1/7	169.5	−	−	+	−	o	+	+++	+
25	2.68	37	87.0	−	+	+	−	++	+++	++	++
18	2.87	42	92.0	−	−	+	−	o	+	+	+++
11	2.73	43	91.5	−	−	+	−	o	+++	+	+++
7	3.73	47	103.0	−	+	−	−	++	o	++	+
21	2.80	49	107.5	−	−	+	−	o	+	+	++
22	2.36	49	108.0	+	−	−	−	o	o	+++	+
23	2.74	49	108.0	+	−	−	−	o	o	+	+
10	2.84	52	110.5	−	−	+	−	o	++	++	++
14	3.12	52	111.5	−	+	+	−	++	++	+++	++
15	3.11	52	114.0	−	−	+	−	o	+	+++	+
8	2.74	55	122.0	+	−	−	−	o	o	+	+
5	3.26	56	126.0	+	−	−	−	o	o	+++	+
Total incidence of lesions					5	13		5	13	17	20

Total number in series: 22

* Ml. of approximately 3% suspension.

† R.St./28: Received injections thrice weekly.

TABLE II

DETAILS OF SECOND EXPERIMENTAL SERIES AND CONTROLS

Pair no.	Rabbit no.*	Age at start (mo.)	Body wt. at death (kg.)	Duration of Caledon blue injections (wk.)	Total Caledon blue †	Macroscopic			Microscopic				Degree of generalized interstitial pneumonia
										Degree & type of emphysema			
						Normal	Marginal lobule	Vesiculation	Nonemphysematous	Marginal lobule	Vesiculation	Generalized	
	R. St./												
1	64	9	2.53	1	5.0	+	−	−	+	o	o	o	++
	65	9	2.11		o	−	+	−	+	o	o	o	+
2	90	9	2.06	1	4.5	+	−	−	+	o	o	o	+++
	91	9	2.72		o	−	+	−	+	o	o	o	o
3	94	10	2.25	1	¤	+	−	−	−	o	+	o	++
	95	10	2.26		4.5	+	−	−	+	o	o	o	o
4	80	12	1.93	2-2/7	9.5	+	−	−	+	o	o	o	+++
	81	12	1.75		o	−	+	−	−	++	o	++	++
5	86	9	2.02	3	15.0	+	−	−	+	o	o	o	+++
	87	9	2.36		o	+	−	−	+	o	o	o	o
6	56	9	2.56	4-2/7	22.5	−	−	+	−	o	++	o	+++
	57	9	2.89		o	+	−	−	+	o	o	o	+
7	110	10	2.66	5-3/7	26.5	−	+	+	−	++	+	+++	++
	111	10	2.29		o	−	−	+	−	o	+	+	++
8	44	10	3.01	6	31.5	+	−	−	+	o	o	¤	++
	45	10	3.04		o	+	−	−	+	o	o	o	o
9	46	11	1.59	6	31.5	+	−	−	−	o	o	+	++
	47	11	1.62		o	+	−	−	+	o	o	o	+
10	78	12	2.65	7	35.5	−	−	+	−	o	++	o	+++
	79	12	2.57		o	−	+	−	+	o	o	o	++
11	104	7	2.06	8	40.0	−	−	+	−	o	+	¤	++
	105	7	2.32		o	+	−	−	+	o	o	o	++
12	48	10	2.33	10	50.0	+	−	−	+	o	o	o	+
	49	10	2.01		o	+	−	−	+	o	o	o	+
13	76	8	1.92	15	72.0	−	+	+	−	++	++	+++	o
	77	8	2.05		o	−	−	+	−	¤	+	o	++
14	102	7	2.88	17-4/7	83.5	+	−	−	−	¤	o	++	++

	103	7	3.32		0	+	−	−	+	0	0	0	+
15	106	9	3.04	20	90.5	+	−	−	−	0	0	+++	+
	107	9	2.89		0	+	−	−	+	0	0	0	+++
16	108	7	2.16	20	90.0	+	−	−	−	0	0	+	++
	109	7	2.16		0	+	−	−	+	0	0	0	+
17	52	12	2.80	23-4/7	104.0	−	+	+	−	+++	++	+++	0
	53	12	2.84		0	−	+	−	−	+	0	0	+
18	54	12	2.70	24	116.5	+	−	−	−	0	0	++	0
	55	12	2.82		0	+	−	−	+	0	0	0	+
19	62	9	2.82	24	116.5	−	+	+	−	+++	+++	+++	+
	63	9	2.53		0	−	+	−	−	+	0	+	+
20	70	9	2.08	24	113.5	−	−	+	−	0	+	+++	0
	72	9	2.32	24	114.5	+	−	−	−	0	+	0	0
	71	9	2.00		0	+	−	−	+	0	0	0	+
21	82	11	2.13	24	115.5	+	−	−	+	0	0	0	0
	83	11	2.15		0	+	−	−	+	0	0	0	+++
22	88	9	2.33	24	115.5	+	−	−	−	0	+	++	+
	89	9	2.75		0	−	+	−	−	++	0	0	+
23	92	7	2.87	24	115.5	−	−	+	−	0	+	++	+
	93	7	3.04		0	+	−	−	+	0	0	0	0
24	96	8	2.41	24	115.5	+	−	−	+	0	0	0	0
	97	8	2.67		0	+	−	−	+	0	0	0	++
25	100	7	2.73	24	116.0	−	−	+	−	0	+	0	+
	101	7	2.91		0	+	−	−	+	0	0	0	0
Total incidence of lesions: After Caledon blue (i.v.)							4	10		4	13	12	19
Controls							7	2		4	2	3	19

Total number in series: Experimental = 26
Control = 25

*Odd numbers: controls

†Ml. of approximately 3% suspension. Discrepancies between the duration of injections and the total dosage of Caledon blue were the result of unavoidable interruptions of the weekly or bi-weekly injections.

trations which enlarge and fuse with destruction of the alveolar walls, it is obvious that there is no hard and fast dividing line between "emphysematous" and "normal." However, in the normal lung, while the number of pores is variable, large pores are rare and there is little or no tendency to fusion.

Classed as grade o were lungs in which the alveolar pores were within normal limits as judged from the experience in 155 rabbits previously reported.[35] Lungs where there was not only a greater amount of fenestration than in the arbitrary normal but where there was also obviously enlargement and fusion of the pores were classified as grade + (Figs. 5 and 6). This grade was also applied to lungs where, though individual foci showed a degree of fenestration characteristic of the more severe grades, the lesions had a rather patchy distribution throughout the lungs. Grades ++ and +++ were the more severe cases where the fenestration was gross and obvious in all parts of the lungs (Figs. 7 to 11).

Experimental Results

Details of the incidence of all types of emphysema, duration of injections and amounts of Caledon blue injected are given in Tables I and II.

First Experimental Series

Table III shows the incidence of the various grades of generalized emphysema in this series. Although no strict controls are available, the incidence of generalized emphysema found in the 155 "normal" rabbits previously described[35] is included for comparison. These received no Caledon blue and were divided into 3 age groups: viz., young (5 to 11 weeks old), miscellaneous adults (probably 9 to 18 months old), and old (over 2½ years old). These figures show that the incidence of the

Table III

INCIDENCE OF GENERALIZED EMPHYSEMA IN FIRST EXPERIMENTAL SERIES

Degree of generalized emphysema	After i.v. Caledon blue	Young	Misc. adults	Old
+++	5	0	1	3
++	4	0	2	4
+	8	0	6	4
0	5	20	105	10
Total no. in group	22	20	114	21

various grades of generalized emphysema in the rabbits which received Caledon blue is very much higher than that in the untreated young and miscellaneous adults. However, the incidence in the experimental series is not significantly greater than that in the old rabbits. In view of this, no definite conclusion can be drawn, as the ages of the experimental animals are uncertain. Nevertheless, the results are suggestive.

Second Experimental Series

Table IV shows the incidence of the various grades of generalized emphysema in the experimental animals of this series and in their litter mate controls. There is a greatly increased incidence of generalized emphysema in the animals which received intravenous Caledon blue.

TABLE IV

INCIDENCE OF GENERALIZED EMPHYSEMA IN SECOND EXPERIMENTAL SERIES

Degree of generalized emphysema	After i.v. Caledon blue	Controls (litter mates)
+++	6	0
++	4	1
+	2	2
0	14	22
Total no. in group	26	25

The results were analyzed by the exact factorial method of Fisher [36] in the following manner: (1) All grades of emphysema were pooled in the injected and control groups respectively, and the incidence of emphysema compared with that of the 0 grade in the two groups. This gave $P = 0.008$. (2) The ++ and +++ grades of emphysema were pooled in the injected and the control groups respectively, and the incidence compared with that of the pooled grades 0 and + in the two groups. This gave $P = 0.003$. Thus the increased incidence of emphysema in the group receiving injections is clearly significant, and it therefore can be concluded that the intravenous Caledon blue has, in fact, produced experimental emphysema.

Influence of Duration of Caledon Blue Injections

Table V shows that the incidence of generalized emphysema increases as the duration of the injections, and hence the amount of Caledon blue, increases. This supports the view that the Caledon blue has produced the emphysema found in the injected animals.

Mode of Action of Caledon Blue

The experiments were performed on the theoretical basis that chronic emphysema should be regarded as an atrophy of lung tissue and that interference with blood supply might produce such an atrophy. Insofar as emphysema has been produced by the introduction of a particulate substance into the pulmonary vessels, the experimental results appear

to substantiate the hypothesis. However, there are other ways in which the Caledon blue might have acted.

There is no indication that fibrosis is produced, and this definitely is not the mechanism involved. Infarction does not occur, and there is no evidence that the emphysema is of the compensatory type. The Caledon

TABLE V

DURATION OF CALEDON BLUE INJECTIONS AND DEGREE OF GENERALIZED EMPHYSEMA

Degree of generalized emphysema	Duration of Caledon blue injections (wk.)						
	First experimental series				Second experimental series		
	1–3	4–11	12–23	24–56	1–3	4–11	12–24
+++	0	0	0	5	0	1	5
++	0	0	1	3	0	0	4
+	0	1	2	5	0	1	1
0	2	3	0	0	5	5	4
Total no. in group	2	4	3	13	5	7	14

blue produces only a slight histiocytic response, which bears no relation to the anatomic distribution of the emphysema, and there is nothing to suggest that the emphysema is the direct result of inflammation or the mere mechanical presence of the Caledon blue particles. It is possible that the particles act as irritants and reflexly alter the mechanics of respiration. However, the only evidence in support of this is the fact that, especially during the first few injections, about half of the animals exhibited a transient tachypnea. This lasted for only 2 or 3 minutes; then the respirations became normal and remained so. This transitory tachypnea was not a constant feature and was rare after an animal had been established on routine injections. Between injections, the animals were clinically normal, and ausculation of the chest never gave any hint of bronchial spasm or increased bronchial secretions. Binger and colleagues,[87,88] in 1924 and 1927, made a careful study of the mechanisms involved in the production of transitory tachypnea in experimental pulmonary embolism in dogs. They concluded that reflex irritation was not the mechanism involved and that the tachypnea was directly related to the amount of vascular obstruction produced.

The suggestion remains, therefore, that Caledon blue acts by causing the obstruction of large numbers of small blood vessels in the lungs, thereby producing an ischemic atrophy of lung tissue—chronic pulmonary emphysema.

GENERAL DISCUSSION

If it can be accepted that Caledon blue R.C., in the absence of distention, produced the experimental emphysema by causing tissue is-

chemia, this affords strong support for the view that chronic emphysema should be regarded as an ischemic atrophy of lung tissue. As was shown in the historical review,[34] this idea is an old one but has received relatively little attention.

The present experiments do not provide an explanation of how tissue ischemia is produced in human cases, but it appears that there are two main ways in which this might occur:

(1) Increased intra-alveolar pressure, produced by any one of the recognized remote mechanisms such as bronchial obstruction or coughing, could directly compress the capillaries which lie unsupported in the delicate alveolar septums and are exposed, on either side, to the intra-alveolar pressure. The upper limit of normal pulmonary arterial pressure is given as 30 mm. of Hg by Cournand,[39] while the upper limit of normal pulmonary "capillary" pressure is stated to be 15 mm. of Hg by Dexter and associates.[40] Estimates of intrathoracic pressure during coughing considerably exceed these levels and may reach as high as + 250 mm. of Hg according to Sharpey-Shafer.[41] Such figures suggest that direct capillary occlusion could arise in diseased states known to be associated with the development of chronic emphysema. In addition to direct occlusion of capillaries by this means, it seems likely that linear stretching or distortion of the vessels, in the course of distention, could also contribute to the production of ischemia.

(2) Pulmonary or bronchial inflammation could interfere with the blood supply of the affected parts of the lungs either by producing endarteritis, as was suggested by Korol,[42,43] or by direct destruction of minute blood vessels. Christie[44] and Whitfield,[45] among others, have pointed out that clinically the severity of the emphysema cannot always be correlated with the severity of the bronchitis or cough. It is possible that ischemia of inflammatory origin, even in the absence of distensive forces, may explain such cases. In this connection it should be remembered that the anatomic studies of Orsós,[46,47] Letulle,[48] Antoniazzi,[49,50] and Bezançon and Delarue[51] led them to believe that inflammatory changes were an integral part of the emphysematous process.

It is felt, as was tentatively suggested by Rindfleisch[52] in 1871, that adoption of the ischemic theory of the intimate pathogenesis of chronic emphysema would help to reconcile the numerous mechanical theories of remote pathogenesis with each other and also with the apparently conflicting nutritional views. Consideration of the known remote pathogenetic factors shows that the views expressed above can be applied equally well to both hypertrophic and compensatory emphysema. In the case of the latter, it might well be, as was suggested by Korol[42] in 1938, that inflammatory vascular changes are more important than mechanical

effects *per se*. As was pointed out in the historical review,[84] there is no systematically documented evidence to prove the existence of "pure" senile emphysema as opposed to emphysema in the lungs of the elderly due to remote causes operative in all age groups. But if such an emphysema exists, it is possible that senile changes in the vascular tree might be important in its genesis.

Other types of chronic emphysema, including the focal emphysema described in the simple pneumoconiosis of coal miners and workers by Heppleston[53,54] have been excluded from consideration. However, it is suggested that ischemia resulting from vascular occlusion in the course of the pneumoconiotic fibrosis might better explain the localization of the focal emphysema around the respiratory bronchioles than disturbance of air flow at this level.

In conclusion, it is felt that this outlook provides a means of reconciling many apparently conflicting views on the etiology and pathogenesis of chronic vesicular emphysema and is in keeping with the basic histologic identity of the lesions in all forms of the disease. Further, a case can be made for ceasing to regard emphysema as a disease in its own right. Pathologically, chronic emphysema should be considered as a nonspecific atrophy of lung tissue which can be produced by many remote factors, all of which, however, operate by means of the intimate mechanism of tissue ischemia. Viewed in this light, chronic emphysema is no more an entity than nephrosclerosis or myocardial fibrosis.

Summary

Experimental chronic pulmonary emphysema has been produced in rabbits by the repeated intravenous injection of an inert particulate dyestuff, Caledon blue R.C. The experimental lesions are identical to those of spontaneous pulmonary emphysema in rabbits and to human chronic emphysema.

It is considered that the Caledon blue R.C. acted by obstructing large numbers of pulmonary blood vessels, thereby causing ischemia. If this is accepted, the experiments afford direct support for the view which considers that chronic emphysema is an ischemic atrophy of lung tissue.

The mechanisms which might be operative in human emphysema are discussed and it is suggested that the ischemic theory of intimate pathogenesis provides a means of reconciling the many varied views on the nature and pathogenesis of chronic pulmonary emphysema.

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Messrs. Imperial Chemical (Pharmaceuticals) Ltd., Wimslow, Cheshire, England, very kindly supplied the Caledon blue R.C.

[*Illustrations follow*]

Legends for Figures

All microscopic-sections have been cut at 100 μ. Sections were stained with hemalum and eosin.

Fig. 1. Vesiculation in experimental emphysema. Scale in mm.

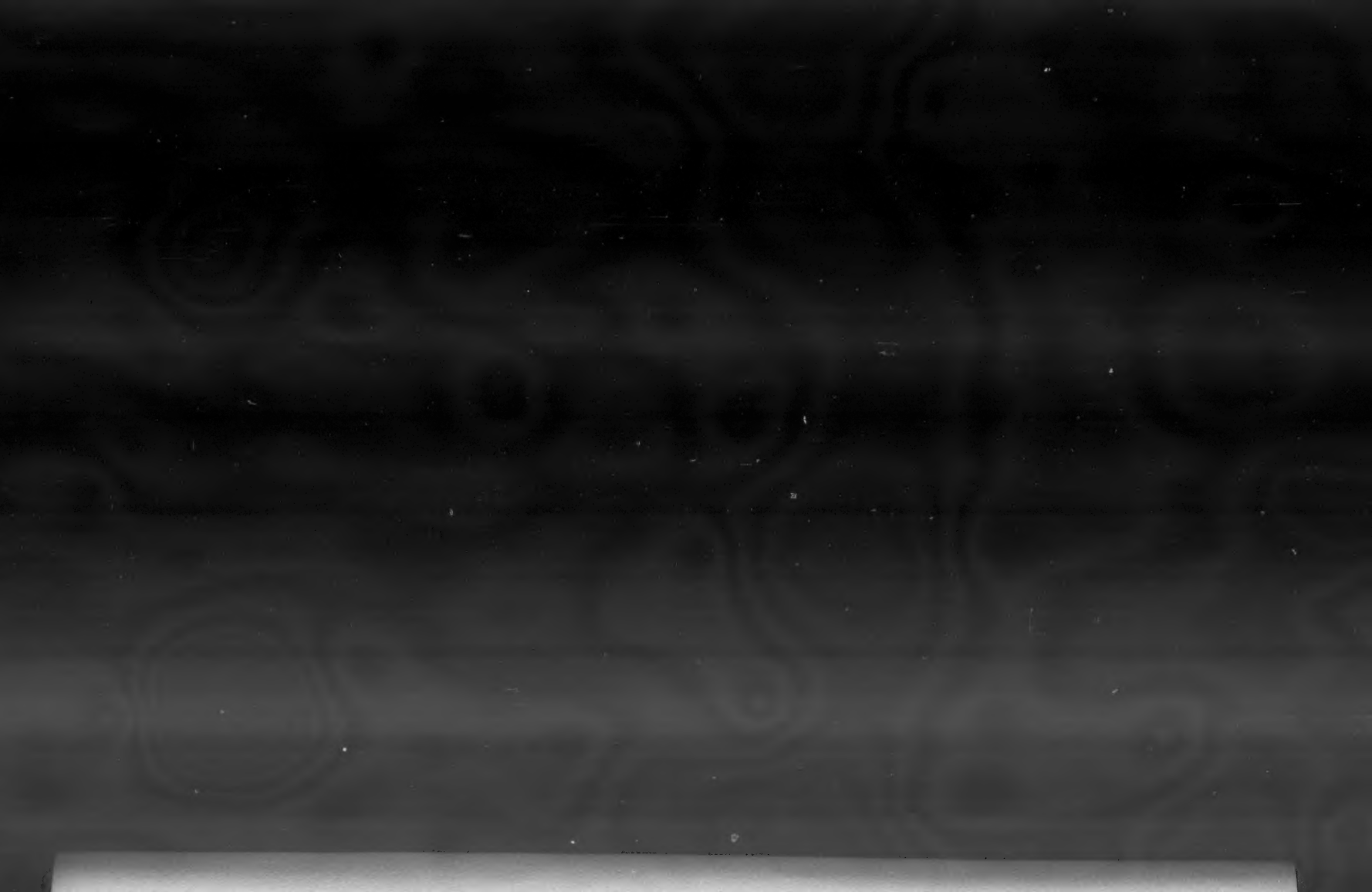
Fig. 2. Fenestration in human chronic vesicular emphysema. × 60.

Fig. 3. Fenestration in emphysematous alveolar septum in experimental emphysema. The large black masses are aggregates of Caledon blue R.C. in vessels. × 480.

Fig. 4. Vesiculation in experimental emphysema, showing loss of alveolar septums, fenestration in septums between alveolar ducts, and Caledon blue R.C. aggregates in the vessels. × 30.

Fig. 5. Fenestration in experimental generalized emphysema grade +. × 60.

Fig. 6. Normal litter mate control for comparison with Figure 5. × 60.



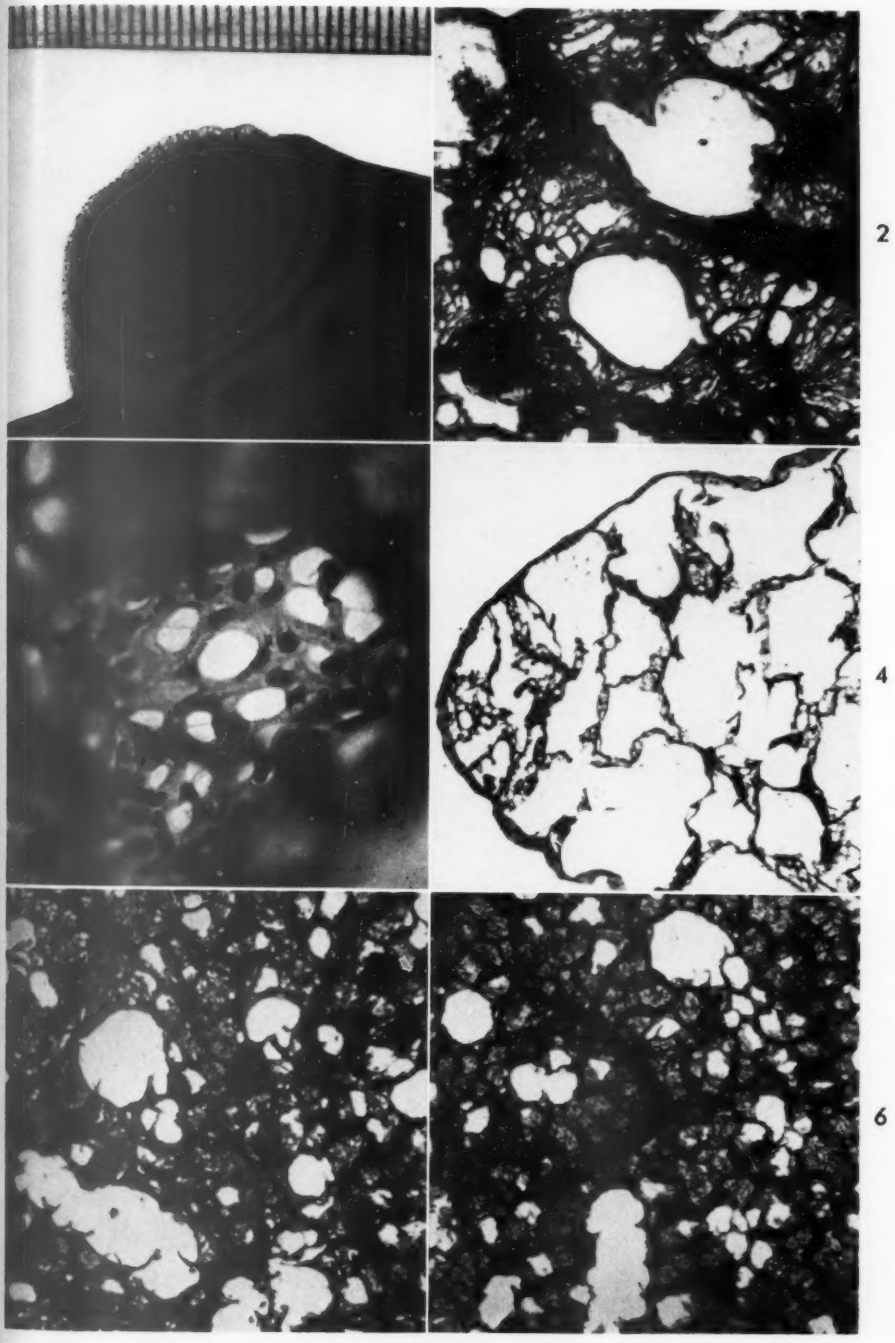

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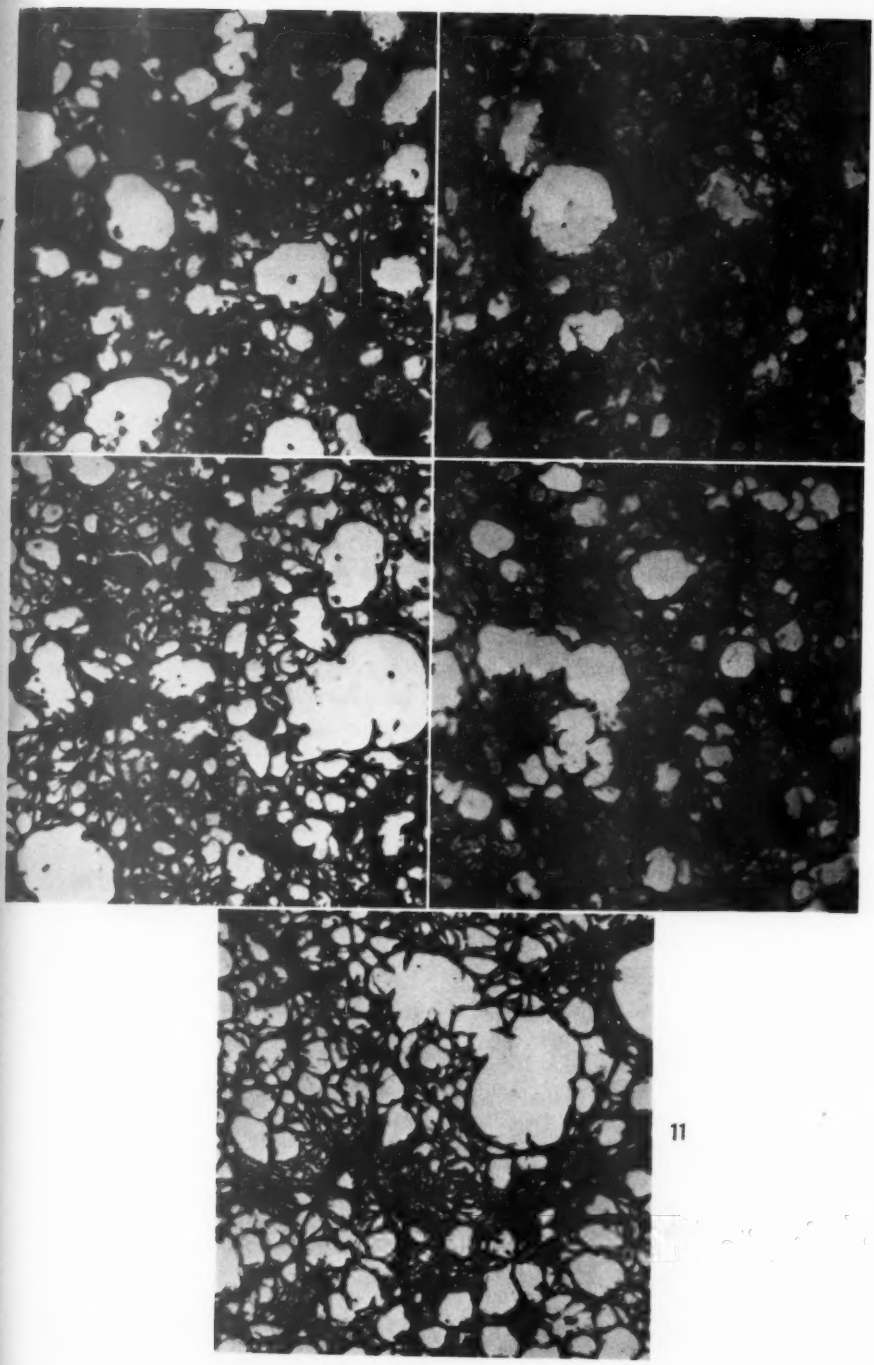
FIG. 7. One of the least fenestrated areas in experimental generalized emphysema grade +++. × 60.

FIG. 8. Normal litter mate control for comparison with Figure 7.

FIG. 9. One of the most fenestrated areas in experimental generalized emphysema grade +++. × 60.

FIG. 10. Normal litter mate control for comparison with Figure 9. × 60.

FIG. 11. Spontaneous generalized emphysema grade +++ in an old rabbit, for comparison with the experimental lesion. × 60.



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ELECTRON MICROSCOPIC OBSERVATIONS OF THE SPLEEN DURING THE INDUCTION OF EXPERIMENTAL AMYLOIDOSIS IN THE RABBIT

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Amyloidosis has been the subject of clinical and pathologic reports for over 100 years. Despite this, the nature of amyloid, its exact areas of localization, and the pathogenesis of the disease remain unknown. A disorder in experimental animals, similar in almost all respects to secondary human amyloidosis, can be produced by a number of techniques. In the past several years, multiple subcutaneous casein injections have been demonstrated to cause amyloidosis in a reproducible manner in the spleen, kidney, and liver of rabbits.[1] The organ in which the lesion was first observed was invariably the spleen. Serial splenic biopsy specimens and sacrifice of animals at specific intervals demonstrated that lesions were occasionally seen at 2 months, whereas after 5 months of continued injections, amyloidosis of the spleen was always present. The histologic appearance and staining reactions were in every way similar to the lesions of secondary human amyloidosis. The amyloid appeared to be deposited in close relationship to the connective tissue beneath the splenic sinuses.

An electron microscopic investigation of the fine structure in splenic amyloidosis was undertaken in order to study (a) the alterations in the reticuloendothelial system during the inception of the disorder; (b) the fine structure of amyloid substance; and (c) the relationship of amyloid to the basement membrane of splenic sinuses. The predictable time sequence and reproducible nature of the experimental disease facilitated this study.

MATERIAL

Amyloid was produced in 12 New Zealand white rabbits by subcutaneous injections of 5 cc. of 10 per cent casein suspension twice weekly for 1 to 6 months. The animals were maintained on a standard diet of Purina Rabbit Chow and water. They were sacrificed at 2, 4, and 6 months, along with untreated rabbits. Portions of spleen, 1 to

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2 cu. mm., were immediately fixed at 0° C. in Dalton's solution (potassium dichromate, 1 per cent; osmium tetroxide, 1 per cent; NaCl, 0.85 per cent) at pH 6.4 [3] or buffered sucrose osmium tetroxide at pH 7.45.[3] The tissues were dehydrated in 70 per cent, 95 per cent and absolute methyl alcohol, embedded in n-butyl methacrylate and polymerized with benzoyl peroxide (15 mg. per ml. of methacrylate) at 60° C. overnight. Specimens of spleen were also fixed in neutral formalin and embedded in paraffin for conventional and polarization microscopy.

The amyloid-laden spleen of one rabbit was fixed according to the procedure of Selby [4] to accentuate fibrous structure. After fixation in buffered osmium tetroxide for 4 hours, the tissue was placed overnight in 1 per cent phosphotungstic acid in phthalate buffer at pH 5.4. It was then dehydrated through alcohols containing 1 per cent phosphotungstic acid.

Sections of the spleens of all rabbits were cut for conventional microscopy and stained with hematoxylin and eosin, Congo red, crystal violet, van Gieson and periodic acid-Schiff (PAS) reagents. The silver stain of Gomori-Bielschowsky was applied to several specimens. Sections were also examined for birefringence in the polarizing microscope. Successive thick (2 to 3 μ) and thin (250 to 500 Å) sections were cut on a Porter-Blum microtome. The thick sections were treated with xylene overnight to remove the methacrylate and stained with PAS and hematoxylin for light microscopy. Thin sections were examined in an RCA EMU 2E electron microscope. Initial magnifications were from 2,400 to 15,000 times.

Results

Light Microscopy

When compared to untreated animals, the spleens of the rabbits given casein injections for 2 months (Fig. 1) showed an apparent increase of cell concentration (especially plasma cells) in the red pulp. There was no evidence of amyloid, i.e., negative Congo red stain, lack of metachromasia and no abnormal eosinophilic hyaline deposit. In the "thick" osmic acid-fixed sections stained by the PAS method after removal of methacrylate, the subendothelial connective tissue (reticulum) was found to be thickened. There were a few cells present containing PAS-positive granules.

In the rabbits with longer courses of casein injections (4 and 6 months), large amounts of amyloid were present. The earliest accumulations appeared in the marginal zones between white and red pulp (Fig. 2), but in the more massive lesion, amyloid was found throughout the organ, sparing only the white pulp. The formalin-fixed amyloid was eosinophilic; it bound Congo red, was metachromatic with crystal violet, and had a structureless or "hyaline" appearance. Following this form of fixation, endothelial cells were sparse and poorly preserved in the red pulp. No cellular reaction to the amyloid was observed. The deposit exhibited minimal blue-green autofluorescence and moderate red fluorescence after Congo red staining when viewed in ultraviolet light at 365 mμ. Birefringence of the unstained material was faint, but a moderately strong positive birefringence in the direction of the long axis of the

deposit was noted in amyloid stained with Congo red. Amyloid had a brown hue with the Gomori-Bielschowsky stain, while the basement membrane was black.

The portions of spleen fixed in osmium tetroxide showed thickening and irregularity of the basement membrane on PAS staining. The amyloid substance was slightly PAS-positive. The fenestrated basement membrane (heavily PAS-positive) could still be observed outlining sinuses and apparently was distinct from the amyloid which was present as nodose thickenings (Fig. 2). No structural organization was observed in the amyloid at this magnification (1,200 times).

Electron Microscopic Observations

The untreated animals displayed no abnormalities in splenic architecture. As previously demonstrated, the venous sinuses of the red pulp had a characteristic structure.[5] This consisted of a complex but regular structural sequence made up of splenic sinus lumen, sinus endothelial cell, basement membrane ("reticulum"), endothelial cell, and lumen of the cord. When the lumen was patent, a frank sinus was seen, and when it was collapsed or the endothelium altered, the structure took on the appearance of a Billroth cord. The lumen endothelial surface was complex and irregular due to cytoplasmic protrusions into the sinus. The basement membrane, subjacent to the endothelial cell, had a finely granular appearance.

The most marked change in the fine structure of the spleen after 2 months of casein injections was widening of the basement membrane. Its substance was not visibly altered at this time. It presented occasional nodular thickenings covered by endothelium and was rarely in direct contact with the lumen of the sinus. No fibrils were observed in this connective tissue. The endothelial cells were not altered. Complex protrusions of cytoplasm into the lumen of the sinus and the generally irregular contours of the cells were apparent. Cellular inclusion bodies were not altered. The numbers of plasma cells in the sinuses appeared to be slightly increased. They had many pseudopods as well as a characteristic pattern of endoplasmic reticulum.[6]

After 4 and 6 months of casein injections, material identifiable as amyloid by light microscopy was observed with the electron microscope (Figs. 3 to 5). Under relatively low magnification, it was immediately apparent that there was far more cellularity than had been appreciated with light microscopy. In the sinuses, many red blood cells, heterophils, and plasma cells were observed.

The amyloid appeared to be somewhat granular. It was located in the subendothelial region, and could not always be definitely distinguished

from basement membrane, although the former was more coarse and mottled. A fine filamentous character in the amyloid was apparent, however, at higher magnification (Fig. 6). The bands of amyloid were much thicker than normal basement membrane, but like the latter in most areas they were in contact with the endothelial cells.

The endothelial cells, though occasionally compressed, and with slender processes reaching into the amyloid, possessed a remarkably well preserved cytoplasmic fine structure. Mitochondria with well defined cristas, vacuoles, dark granules, endoplasmic reticulum and nuclear structure were not altered. Occasionally, these cells appeared to be breaking away from the subendothelial amyloid into the sinus lumen. The endothelial cell surface bordering on the amyloid was occasionally filled with clear vacuoles similar to those seen in endothelium with marked phagocytic and pinocytotic activity (Fig. 6).

The amyloid-laden spleens clearly demonstrated the repeating pattern of lumen, endothelial cell, amyloid (in place of or in addition to basement membrane), endothelial cell, lumen, etc. (Fig. 3). The plasma cells in the sinuses were remarkable only in the multiplicity of pseudopods and adaptability of the cell surface to the complex and irregular sinus lumens.

In the spleens with little amyloid, the substance appeared to be consistently separated from the sinus lumen by endothelial cytoplasm. In massive amyloidosis, occasional direct contact of the amyloid with the sinus lumen was observed when the endothelial cytoplasm was very attenuated or the cell itself appeared to have broken off into the general circulation (Fig. 4).

At high magnifications, several elements were seen within the amyloid (Figs. 7 to 9): (a) the complex infolding and delicate extensions of endothelial cytoplasm; (b) fine granules of indeterminate nature; (c) fine filaments criss-crossing in all directions through the amyloid or in parallel array. The latter were more clearly seen in their filamentous form in areas adjacent to the endothelium.

The rabbit spleen fixed according to the technique of Selby showed some distortion and destruction of the cellular elements. The amyloid itself, however, was found to contain long filaments, measuring under 300 Å in thickness and of lengths ranging up to 1,500 Å (Fig. 10).

Discussion

When amyloidosis is produced in rabbits by casein injections, the spleen is the earliest site of localization. Subsequently other parenchymal organs become involved in a predictable fashion. The actual source and nature of the amyloid has never been clearly delineated. It

has been variously suggested to (a) be of intracellular origin and dislodged from within reticuloendothelial cells into the extracellular spaces[7]; (b) originate from circulating blood, representing either precipitated plasma proteins[8] or an antigen-antibody precipitate[9]; or (c) be deposited in an epicellular fashion,[10] perhaps laid down on preexisting connective tissue fibers.[11] Evidence for each of these theories has been based largely on static pathologic features at the end stages of the disorder or has been derived by deduction based on limited staining or immunologic techniques.

Several morphologic observations are germane to the hypothesis that amyloid is of intracellular origin. In the present investigation, there was no direct evidence of intracellular localization of amyloid by either light or electron microscopy. Although this suggests that amyloid may not be formed intracellularly, it is not at all to be considered proof of this fact, for intracellular precursors of a substance need not necessarily have the same characteristics as the mature moiety. Collagen formation presents an interesting analogy.[12] Electron microscopic observations demonstrated remarkably normal endothelial cell organelles even in the face of massive deposits of amyloid. Occasionally (Fig. 6) evidence of increased surface irregularity at the area of contact between cytoplasmic membrane and amyloid was seen, but the significance of this was not manifest. It was observed that the earliest amyloid depositions occurred in the marginal zones of the red pulp just surrounding the lymph follicles. The sinuses here were large, arranged circumferentially about the white pulp, and showed a high degree of phagocytic activity.

In this region, a slight increase in plasma cells was observed in the spleen prior and subsequent to the appearance of amyloid. Although it is believed by some that this plus pyroninophilia indicate the intracellular production of amyloid,[13] the present work does not necessarily add to this concept.

The relationship of amyloid to the vascular system is of interest. The earliest deposits of amyloid observed were consistently separated from the sinus vascular spaces by endothelial cells. When massive deposits were examined, amyloid occasionally appeared to be in direct contact with the circulating blood. It is possible that the few instances in which this was encountered were attributable to artifact of fixation or sectioning. It would appear that if amyloid were deposited from the circulating blood, it would have to traverse the endothelial cell itself or pass through the intercellular space to localize in the subendothelial area.

Any consideration of the theory that amyloid accumulates in the con-

nective tissue in an epicellular or subendothelial fashion requires further definition of the connective tissue components. Unfortunately, with the advent of more specialized staining techniques and electron microscopy, terminology with respect to basement membrane, reticulin, reticulum and the like have taken on various meanings. Clarity in descriptive terminology for structures observed with the light and electron microscopes is vital to the present observations since the earliest alteration in the fine structure of the rabbit spleen was a thickening in the region of the basement membrane.

In the present discussion, it is proposed to use the term basement membrane in its classic histologic sense; that is, as a thin membrane, with an affinity for silver or PAS stain, lying directly beneath an epithelial or endothelial surface. The close relationship of basement membrane to ground substance of the connective tissue has been discussed at great length by Gersh and Catchpole.[14]

The basement membrane as described by electron microscope observers is usually regarded as a thin, finely granular layer beneath epithelium or endothelium. However, Weiss and Ferris demonstrated that the basement lamella in amphibian skin was made up of a delicate meshwork of fibrils embedded in a homogeneous ground substance.[15] Recently Salpeter and Singer noted the same structured basement lamella, but also described a thin granular membrane between it and the overlying epidermal cells. They suggested the term "adepithelial" or "adepidermal" membrane for this structure so as to avoid confusion with "basement membrane" as used in the classical histologic sense.[16]

As the amyloid appeared in its completely developed form, it was found to occupy the subendothelial area more fully where previously only the basement membrane had lain. The amyloid was more coarsely granular, and in phosphotungstic acid-stained preparations contained fine threadlike filaments.

It is of interest to speculate whether the amyloid completely replaced or was deposited in the basement membrane, or, indeed, whether amyloid itself is a "basement membrane-like" material. Dahlin[17] and subsequent authors, in their descriptions of secondary human amyloidosis, have noted its predilection for formation in or about basement membranes. Missmahl, influenced by its anisotropy, suggested that it was deposited on a matrix of collagen.[11] Since the earliest change seen in the present investigations was a thickening in the fine structure of the basement membrane, it was apparent that light microscopy might be of value in differentiating amyloid from normal splenic supporting tissues.

Here again, however, terminology must be standardized. Mall, in 1896, described 3 fibrous components in the connective tissue, namely,

white connective tissue (collagen), elastin, and the reticulum.[18] The last element, which he distinguished from collagen by its lack of gelation on boiling, constituted the finest supporting network of fibrils in the spleen and other organs. Subsequent authors used the term "reticulin" synonymously with "reticulum." This should not be confused with endoplasmic reticulum, an intracellular component of entirely different character, discovered with the electron microscope.[19,20] Starting in the late 1920's, there appeared a series of papers in which attempts were made to ascertain the relationship of reticulin to collagen. This subject has been extensively reviewed by Robb-Smith,[21,22] who found a lack of uniformity and purity in reticulin examined by various techniques in different laboratories. At the present time, the problem has not been solved, but many authors tentatively accept the idea that there are at least 2 types of reticulin.[12,22] One, seen in developing connective tissue, precedes the appearance of thicker collagen fibrils and appears to represent thin collagen fibrils. The other "reticulin" is associated with the basement membrane of parenchymatous organs and lies beneath the epithelium. The latter takes a silver stain and is strongly PAS-positive. It is this form of reticulin (the original reticulum of Mall) with which we are now concerned.

In the spleen, the reticulin, as identified by silver and PAS stains, represents the basement membrane of the splenic sinus. Thus far, no fibers have been identified in the splenic sinus basement membrane by electron microscopy. PAS stains demonstrated the well known fenestrated reticulin in control rabbits (Fig. 1). With the appearance of large amounts of amyloid, PAS-positive material was more widely separated. Amyloid was only faintly PAS-positive (Fig. 2).

The Gomori-Bielchowsky silver stain demonstrated the classic reticular architectural pattern in normal spleen. In those laden with amyloid, the black silver-staining reticulin was preserved in many areas but obscured in regions with heavy deposits of amyloid. The amyloid itself had a brownish purple hue as opposed to the black stain of the reticulin. In occasional electron micrographs, "basement membrane-like" substance could be distinguished from the amyloid (Figs. 5 and 9). It would seem likely, therefore, on the evidence to date, that the amyloid is deposited in the area of the basement membrane, but, at least in part, it is a distinct structural entity.

The intrinsic structure of amyloid was complex and was composed of small granules and fine filaments. The latter in certain sections had a beaded appearance. It is possible that all the electron dense material was finely filamentous and the appearance of granularity was due to the delicate and possibly beaded nature of the threads. Tissue fixed in

buffered phthalate overnight and stained with phosphotungstic acid demonstrated more clear-cut filaments in the amyloid. Their dimensions were consistent with the fibrous material noted in experimental renal amyloid and in primary and secondary human amyloidosis.[23,24,25] The filamentous components would appear to explain the property of positive birefringence with respect to the long axis of the deposit, when it was viewed through the polarizing microscope.

Summary

1. The spleens of rabbits with casein-induced amyloidosis were examined by light and electron microscopy at various stages in development.

2. The amyloid so induced bound Congo red, exhibited metachromasia with methyl violet, and was eosinophilic. It appeared initially in the marginal zone of the red pulp and culminated in massive replacement of red pulp and eventually white pulp.

3. By electron microscopy, prior to the development of clear-cut amyloidosis, thickening of the subendothelial basement membrane was the first change observed. Amyloid accumulated progressively, forming large nodules separated by cytoplasmic processes. Endothelial cells were stretched over the masses of amyloid and separated it from direct contact with the blood stream. Intracellular organelles were unaltered.

4. The amyloid itself had a granular and fine filamentous appearance, and contained many cytoplasmic projections.

5. Although it first accumulated in the region of the basement membrane, it seemed, in part at least, to be distinct from this structure.

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[*Illustrations follow*]

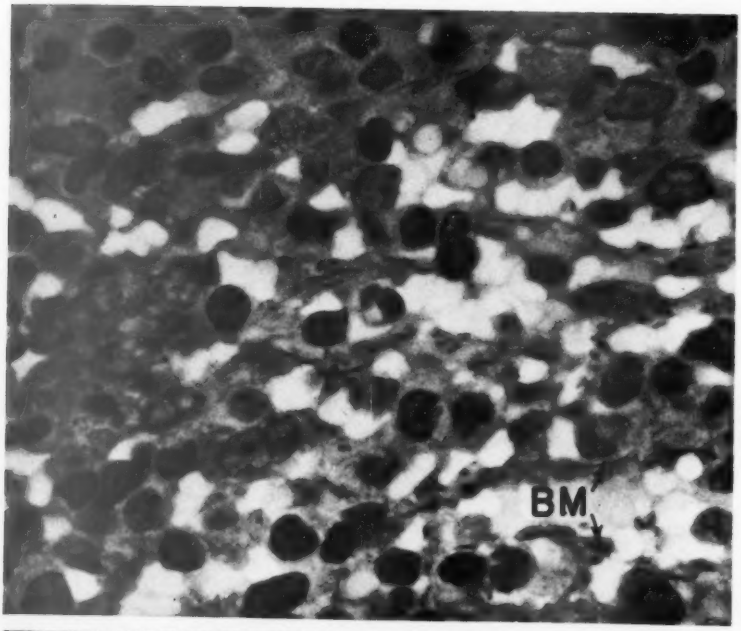
Legends for Figures

Key:

Am	= amyloid	MZ	= marginal zone
BM	= basement membrane	NUC	= nucleus
CYT	= cytoplasm	RBC	= red blood cell
END	= endothelial cell	RP	= red pulp
END. cyt.	= endothelial cytoplasm	V	= "vacuolelike" structures
L	= lumen	WP	= white pulp
MIT	= mitochondria	X	= unidentified cell

Fig. 1. The red pulp of a control rabbit spleen. Sinuses contain unstained red cells. Endothelial cells lining the lumen protrude into it in an irregular fashion. An interrupted basement membrane lies under the endothelium. Dalton fixative; methacrylate embedded; sectioned at approximately 3 μ. Periodic acid-Schiff (PAS) stain. × 1300.

Fig. 2. The red pulp of the spleen in a rabbit given casein injections for 5 months. Marginal zone between the white pulp and red pulp shows accumulation of amyloid partially occluding the sinuses. The amyloid appears to lie beneath the endothelium. The basement membrane, though somewhat disrupted, is strongly PAS-positive, while the amyloid is weakly so. Dalton fixative; methacrylate embedded; sectioned at approximately 3 μ. PAS stain. × 1300.



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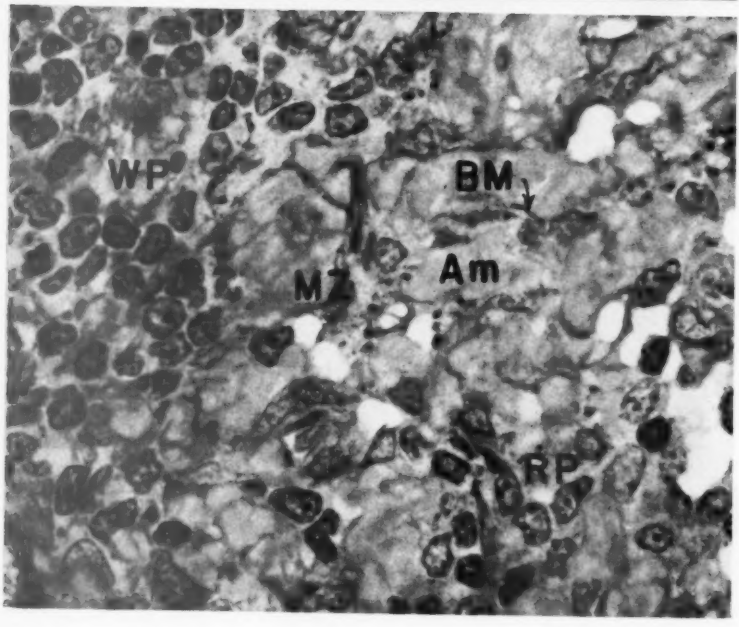

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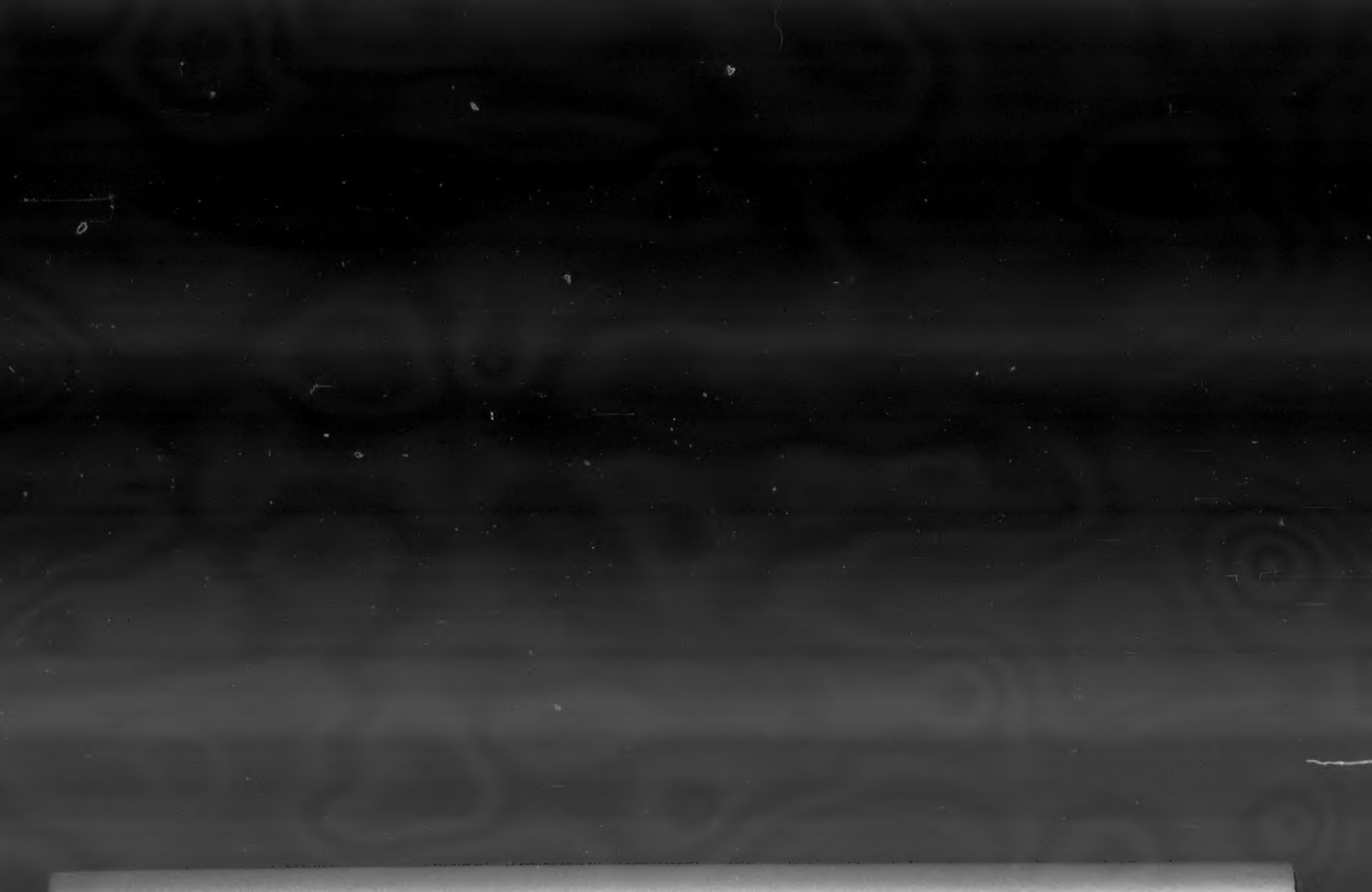
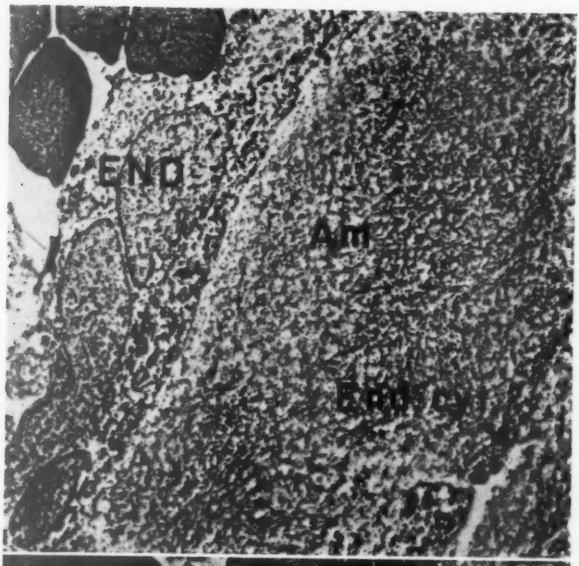
Electron micrographs of the red pulp in the spleen of a rabbit which received casein injections for 5 months. Dalton fixative.

FIG. 3. A sinus containing red cells occupies the upper right. An endothelial cell lies upon amyloid that replaces the basement membrane. A cytoplasmic process of an endothelial cell is present in the amyloid. × 5000.

FIG. 4. In several areas (arrows), endothelial cytoplasm separating the amyloid from the sinus lumen becomes quite attenuated and possibly broken. It cannot be ascertained definitely whether this direct contact of amyloid with circulating blood is real or due to artifact. × 5500.

FIG. 5. A terminal arterial vessel (with an endothelial lining) is surrounded by a fine basement membrane. The latter is a gray homogeneous structure in close relationship to but distinct from the amyloid. × 5000.





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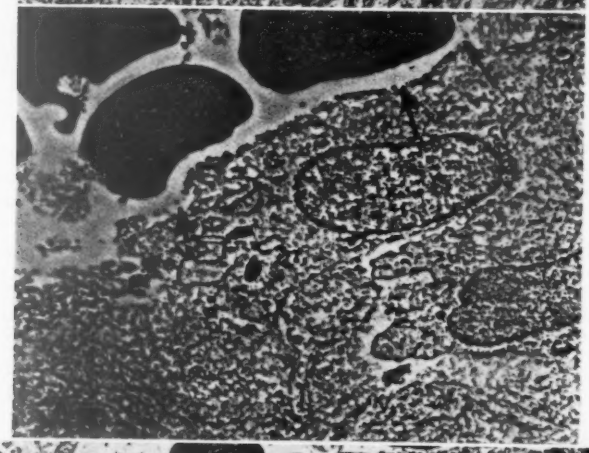

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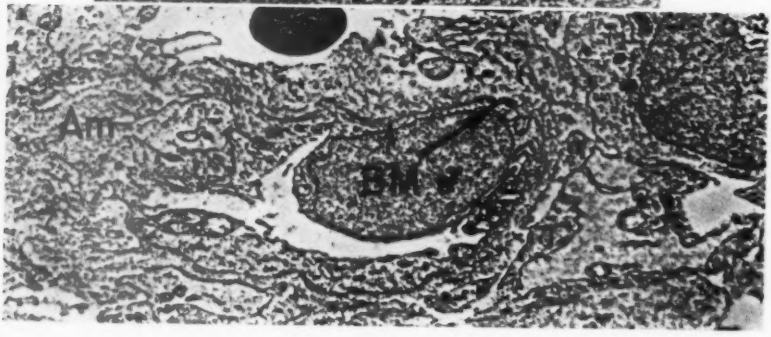

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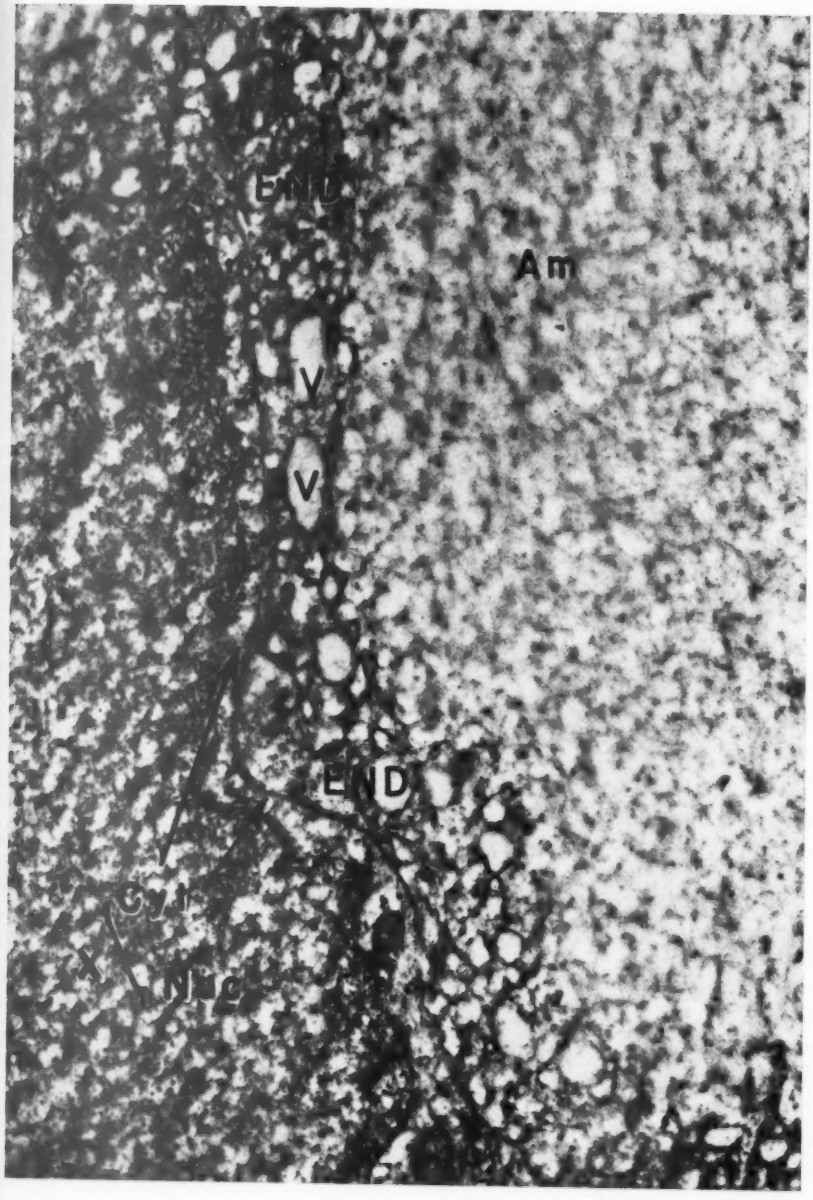
FIG. 6. Red pulp in the spleen of a rabbit that had casein injections for 10 months. In the lower left is the nucleus and cytoplasm of an unidentified cell plus overlapping cytoplasm of an endothelial cell. The border of the endothelial cytoplasm abutting the amyloid has a distinct margin. There are multiple "vacuole-like" structures near this border. These are either true vacuoles or possibly degenerating mitochondria. The amyloid has an almost granular appearance, but fine filaments appear to course through it. Dalton fixative. × 33,000.



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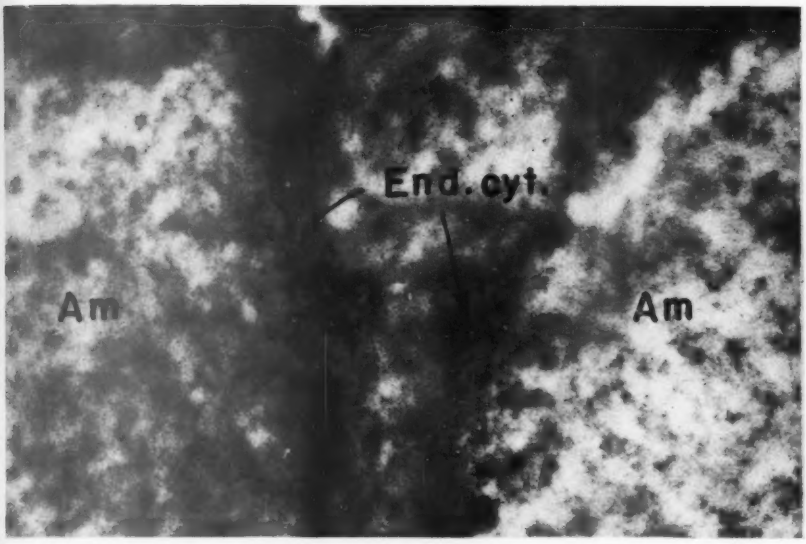
Electron micrographs of the red pulp in the spleen of a rabbit that received casein injections for 10 months. Dalton fixative.

FIG. 7. Two delicate processes of endothelial cytoplasm extend into the substance of the amyloid. The latter is finely granular and has a fluffy appearance although fine threadlike filaments run through it. × 36,000.

FIG. 8. Amyloid is separated from the sinus lumen by endothelial cytoplasm. Cytoplasmic organelles in the latter are all well preserved. The plane of sectioning causes the lumen to appear as a loculated space. × 36,000.



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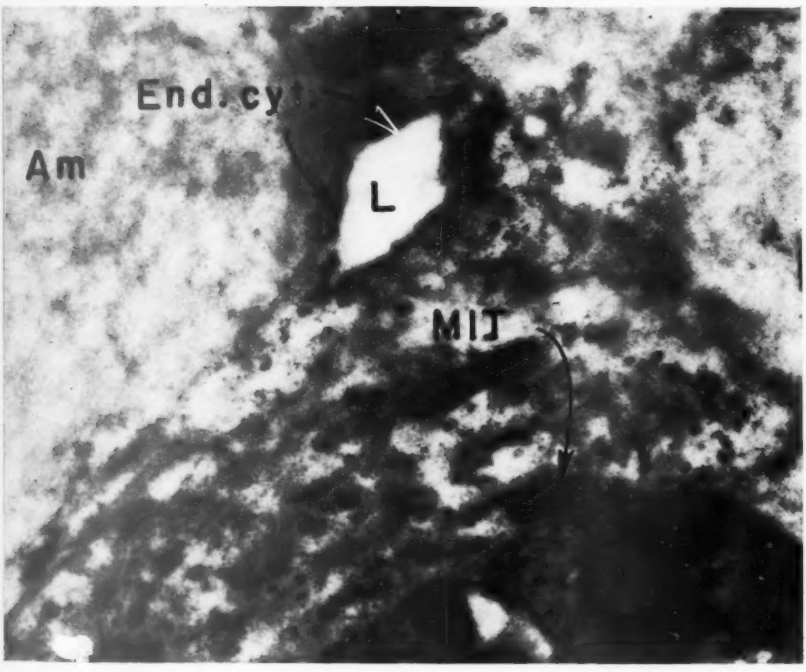

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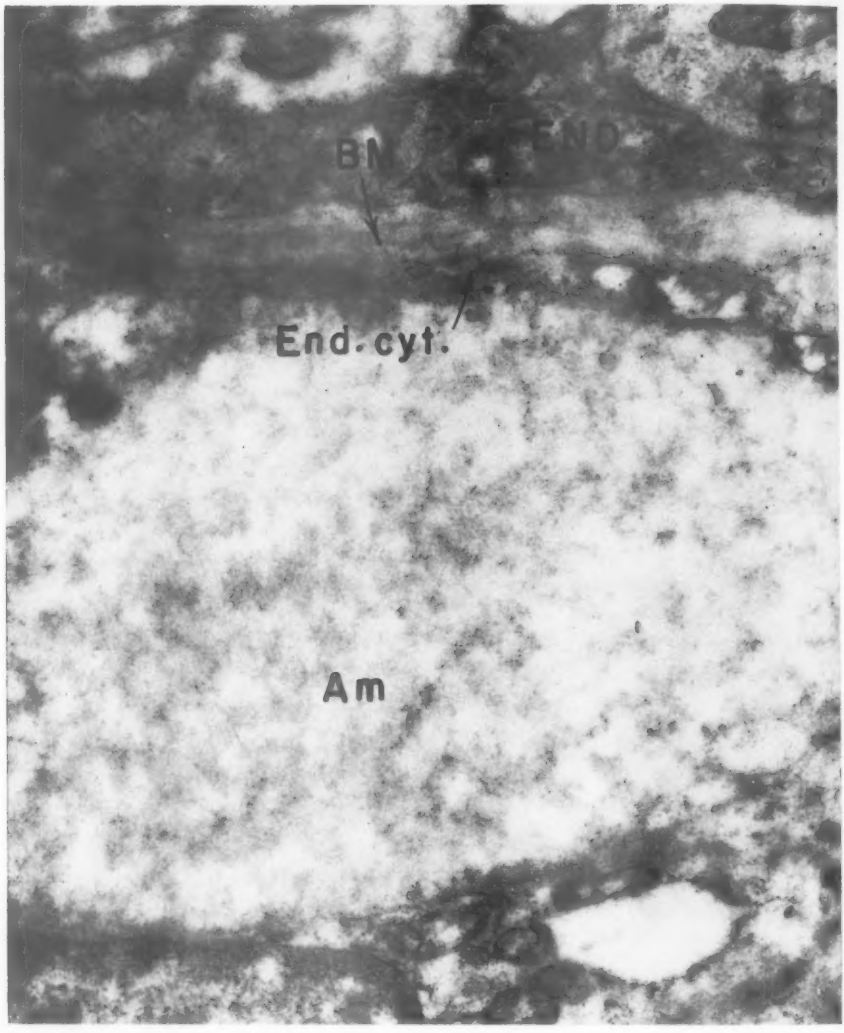

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FIG. 9. Electron micrograph of the red pulp in the spleen of a rabbit that received casein injections for 12 months. Dalton fixative. Amyloid is again found in close relationship to endothelial cytoplasm. Intracellular organelles are well preserved. In the area adjacent to the endothelial cell, "basement membrane-like" substance appears to be distinct from the amyloid. An endothelial cytoplasmic process juts into the amyloid. Fine filaments in amyloid are almost invisible in the photographic reproduction. × 36,000.

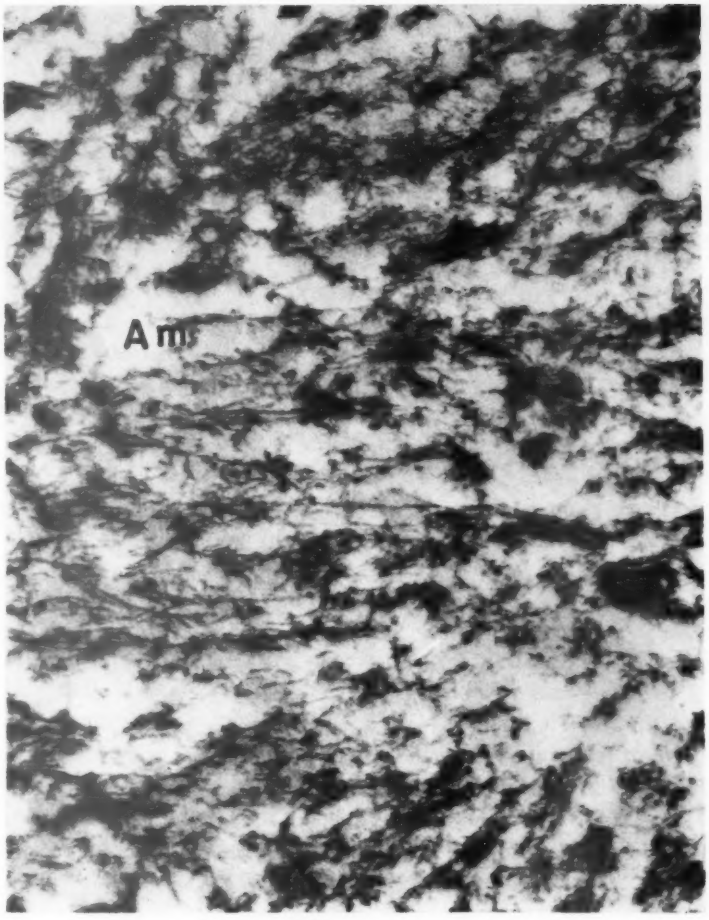

FIG. 10. The red pulp of a rabbit that received casein injections for 3 months. This spleen, after osmium fixation, was immersed in 1 per cent phosphotungstic acid in a phthalate buffer at pH 5.4 overnight, then dehydrated and embedded in methacrylate. Most of the cellular architecture was destroyed, but the fibrous nature of the amyloid deposit is evident. Similar fibrils were not observed in the basement membrane of a control rabbit spleen treated simultaneously in the same manner. × 35,000.

STUDIES ON HEPATITIS IN HAMSTERS INFECTED WITH EQUINE ABORTION VIRUS

III. Nuclear Protein Changes. A Histochemical Study

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Inoculation of the Syrian hamster with equine abortion virus (EAV) causes a rapidly lethal hepatitis which is quite reproducible. A detailed study of the sequential development of the intranuclear inclusion bodies with hematoxylin and eosin and Feulgen stains has been published.[1] Inclusions of various kinds also occur in many other virus infections and have been the subject of numerous investigations. The genetic importance of deoxyribonucleic acid (DNA) has stimulated the use of the Feulgen reaction in these investigations although other methods for the demonstration of DNA, as well as ribonucleic acid (RNA), have been used. Somewhat less attention has been given to the protein nature of virus-induced inclusions, although recent studies have indicated that in human papilloma virus [2] and vaccinia virus [3] infections, the inclusions, which are Feulgen-positive, do contain protein. In adenovirus type 5 infected HeLa cells,[4] intranuclear crystals of nonhistone protein are seen; these are distinct from the intranuclear Feulgen-positive crystals of the virus. The possibility that the EAV inclusion contained appreciable quantities of protein was considered in the present study. Several procedures for the demonstration of proteins were employed, and the observations form the basis of this report.

Material and Methods

A description of the virus, experimental animals utilized and method of inoculation has been documented.[1]

Collection of Tissue and Fixation

The animals were etherized lightly, major blood vessels in the chest cavities were snipped, and the livers were perfused via the portal vein in the cold with physiologic

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saline. A portion of each liver was fixed for 6 to 18 hours in neutral formalin, embedded in paraffin and cut at 6 to 8 μ. The remainder of the livers was employed for other purposes. Liver specimens were harvested at 3, 6, 9, 12 and 15 hours following inoculation, and control tissue was taken from noninoculated animals at each passage.

Histochemical Methods

All sections were stained with hematoxylin and eosin to provide a basis for comparison.

The method of Alfert and Geschwind[8] for basic proteins using fast green FCF at pH 8 was employed. This procedure required extraction of nucleic acids for 15 minutes in 5 per cent trichloroacetic acid (TCA) at 90° C. The sections then were rinsed 3 times in 70 per cent ethanol, once in distilled water, and stained for 30 minutes in a 0.1 per cent solution of fast green FCF adjusted to pH 8 with a 1 per cent solution of sodium hydroxide. The pH was observed throughout the entire staining operation by the continuous use of a pH meter; the solution was stirred gently with a magnetic stirring bar, and the reaction was controlled with NaOH and HCl. Following this, the sections were washed for 5 minutes in distilled water, dehydrated and mounted. The staining operation was carried out at room temperature.

Mercuric bromphenol blue (MBB) was prepared according to the method of Mazia, Brewer and Alfert.[6] Sections were stained for 15 minutes in a 10 per cent aqueous solution of mercuric chloride, and 0.1 per cent bromphenol blue. The sections then were washed for 20 minutes in a 0.5 per cent solution of glacial acetic acid in distilled water, and finally were rinsed for 3 minutes in distilled water alone, dehydrated and mounted. For the demonstration of nucleoproteins, sections were extracted for 15 minutes in 5 per cent TCA at 90° C. in order to remove the nucleic acids. They were then stained as indicated with MBB. Under these conditions nucleoprotein-containing structures stained with an increased intensity when compared to unextracted controls.

Millon's reagent was prepared according to Pearse.[7] Sections were stained at room temperature until the reaction was complete (2½ to 3 hours), rinsed in cold 2 per cent nitric acid, dehydrated, cleared and mounted.

In order that further comparison might be made with earlier work,[1] tissues were fixed in Carnoy's solution (ethanol, chloroform and glacial acetic acid, 6/3/1, v/v/v) for 2 to 3 hours at room temperature and stained by the Feulgen reaction.[1] The specificity of the reaction was checked with deoxyribonuclease (obtained from Worthington Laboratories, Freehold, New Jersey), as described by Kurnick.[8] Sections were incubated at 37° C. for 2 hours in 0.01 M "tris" buffer, pH 7.6, containing 0.02 mg. enzyme per ml. Two sets of controls were prepared; one was incubated in a manner identical to that employed with the treated sections except for the omission of the enzyme from the buffer; the other was hydrolyzed and stained immediately following hydration, with no intervening incubation.

Results

Hematoxylin and Eosin Stain

The sequence in the development of intranuclear inclusions was followed, and was essentially as that previously reported.[1]

Alkaline Fast Green (AFG) Stain

In all sections (Figs. 1, 4 and 7) the cytoplasm, when compared to the nuclei, stained very faintly or not at all. This was to be expected since this stain is considered to be specific for histones and other basic

proteins.* In the normal tissues the nuclear margins and chromatin network were distinct. This was the case with nucleoli as well; many of these stained intensely around the border but not in the center. At 6 hours dark-staining material was occasionally increased around the periphery of the nucleus and the nucleolus. At this time the chromatin network of intermingled dark and pale threads was much more distinct than in comparable hematoxylin and eosin (H-and-E) stained sections. At 12 and 15 hours, peripheral margination of the chromatin was prominent. At the same periods the nuclei contained a pale-staining network without any noticeable inclusion substance. This simulated a chromatin network which ordinarily was not recognizable in infected tissue stained by the Feulgen and H-and-E methods. It is worthy of note that no inclusion as such was recognizable with this stain during any stage of infection. It should be emphasized that with H-and-E and Feulgen stains, inclusions were infrequent at 6 hours. At 9 hours and thereafter, however, practically every parenchymal cell contained an intranuclear inclusion.[1]

Mercuric Bromphenol Blue Stain

Nuclei and cytoplasm were indistinct in normal tissues as well as in those removed 3 hours after inoculation. At 6 hours, scattered retracted nuclei or inclusions (it was impossible to determine which) were occasionally visualized and were composed of homogeneously staining material (Fig. 5). The cytoplasm and nuclei were darker than in uninfected tissue. There was no major difference between the 6-hour and 12- and 15-hour preparations (Figs. 2, 5 and 8).

When the sections were extracted with hot TCA prior to staining, certain differences emerged (Figs. 3, 6 and 9). The nuclear detail and structures were brought out strikingly in the normal as well as in the infected tissues. Six hours after inoculation, the contrast of nuclear staining, when compared to that in unextracted tissues, was increased noticeably. In most cases the nuclei were homogeneously and deeply stained. At later periods of infection the nuclei either contained typical Class A inclusions[9] or were filled with similar staining substance and were not unlike those in H-and-E and Feulgen preparations.

An unexpected result was noted when the TCA-extracted, MBB-stained (TCA-MBB) sections were compared with unextracted, MBB-stained sections. As predicted, in the normal tissues the intensity of staining of the cytoplasm showed little change after extraction. However, at 6 and 12 hours after inoculation, the cytoplasm stained with a reduced

* The term "basic proteins" is used here to denote an ill-defined group of polypeptides or proteins such as histones, all of which have a high isoelectric point.

intensity in the extracted sections. It appeared that the extraction procedure, which ordinarily would remove nucleic acids, also removed some MBB-staining component peculiar to the infection. This would otherwise have remained in the section throughout the staining procedure. One possibility is that the RNA of the cytoplasm was bound to amino acids or peptides in larger amounts than normal and all were extracted together. Geschwind and Li [10] have shown that certain free amino acids and peptides will bind MBB, but Mazia, Brewer and Alfert [6] suggested that the dye complex would be dissolved away during the washing of preparations. In any event the different morphologic appearance of the cytoplasm in the later stages of infection indicated some change in the cytoplasmic structure.

Millon Stain

The tissue in general was rather pale-staining. The nuclei of normal tissue were indistinct against a pale brown background of cytoplasm. Beginning at the sixth hour of infection and at later intervals, the nuclei contained pale, brown-staining, rather homogeneous material which either filled the nucleus or was arranged as a typical inclusion with a halo. The cytoplasm stained approximately the same here, but was more granular. This was interpreted to indicate that the infected nuclei contained protein. Since the absorption maximum of Millon's-stained material is in the ultraviolet region of the spectrum, the sensitivity of observations made with visible light is lowered, and quantitative differences may therefore be somewhat more difficult to detect. Differences in tyrosine content also may influence such observations.

Feulgen Stain

A detailed description of the sequences in the development of the intranuclear inclusions as demonstrated by the Feulgen reaction has been published.[1] In the present study these observations were confirmed. In order to rule out the possibility that the Feulgen staining of the inclusions was a false positive reaction, deoxyribonuclease (DNase) was employed as described above. The DNase-treated sections failed to stain, while the incubated controls stained with undiminished intensity, indicating the specificity of the reaction.

Discussion

It is apparent that several procedures (AFG, MBB, TCA-MBB) reveal differences in staining qualities from those observed in previous studies utilizing the H-and-E and Feulgen techniques.[1] The AFG pro-

cedure failed to demonstrate inclusions as an accumulation of basic protein. The classical peripheral margination was, however, sharply outlined. An unexpected feature with these stains was the presence of conspicuous chromatinlike material without recognizable inclusions at any stage of infection.

With respect to the basic proteins visualized with AFG in the nucleus, the considerable clumping at 6 hours occurred coincidentally with the intense uniform color of the nucleic acid-extracted nuclei when stained with MBB. With the AFG stain at 6 hours the nucleoli of some cells appeared to have a much thicker layer of basic protein surrounding them than normally. This was similar to the observation of Ohno and Kinosita [11] on the formation of the intranuclear inclusion bodies in contagious canine hepatitis.

It should be noted that MBB-stained tissue following extraction with hot TCA presented unusual findings, most noteworthy early in infection. At 6 hours most of the parenchymal nuclei were filled with dark-staining material which, according to the interpretation of Mazia, Brewer and Alfert,[6] is a nucleoprotein. However, this was not apparent with the H-and-E and Feulgen stains, as these methods demonstrated only an occasional inclusion at this time. At 12 hours and subsequent periods of infection the nuclei showed approximately the same alterations observed with other staining methods.[1] While no independent evidence is available indicating the specificity of this procedure for nucleoproteins, the results of the Millon reaction indicated that the nuclei at 6 hours did contain homogeneously distributed protein. Moreover, the nucleoprotein-containing structures of normal nuclei, the chromosomes and nucleoli, stained intensely with TCA-MBB as would be expected if the method was specific for nucleoproteins.

The question of a relationship between MBB-staining nuclear material in extracted sections with the virus itself must be considered. Several possibilities exist. The material may be a collection of virus particles, virus precursor, a substance not used in virus synthesis, or a mixture of these. Preliminary electron microscope studies [12] have demonstrated that virus particles are seen only in scattered nests in the hepatic parenchymal nuclei of animals sacrificed 12 and 15 hours after inoculation. An examination of published growth curves [1] suggests that virus release from the liver cells has begun to decrease at this period, since the titer in blood does not increase after 12 hours. At 6 hours, however, the synthesis of virus (as indicated by the growth curves) is beginning, and the material seen could easily represent virus or a precursor. Further investigations are in progress in an attempt to elucidate this problem.

Summary

A further study of the morphologic alterations accompanying equine abortion virus-hepatitis in hamsters is reported. Several histochemical techniques were used to demonstrate proteins.

With the alkaline fast green method for histones it was found that while the clumping of the chromatin was visualized quite well, at no stage of the infection were inclusions observed.

Both Millon's reaction and staining with mercuric bromphenol blue (MBB) were used as general protein indications; these showed that the inclusions did contain protein. When sections were first extracted with hot trichloroacetic acid and then stained with mercuric bromphenol blue (TCA-MBB), a striking intranuclear accumulation of densely staining material, presumably nucleoprotein, was seen 6 hours following inoculation in most of the liver parenchymal cells. In striking contrast, only occasional inclusions were seen at this time with the H-and-E and Feulgen stains. At 12 to 15 hours, however, H-and-E, Feulgen and TCA-MBB stained sections all were similar in appearance, with abundant intranuclear inclusions and margination of the chromatin.

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[*Illustrations follow*]

Legends for Figures

Preparations were stained with alkaline fast green (AFG), mercuric bromphenol blue (MBB), or extracted with hot trichloroacetic acid and then stained with mercuric bromphenol blue (TCA-MBB).

Fig. 1. Uninfected control liver. The cytoplasm is stained faintly. Nucleoli are quite distinct and often appear hollow. AFG stain. × 950.

Fig. 2. Uninfected control liver. The nuclear membranes are barely distinguishable and the architecture in general is indistinct. MBB stain. × 1300.

Fig. 3. Uninfected control liver. Extracted with hot trichloroacetic acid and stained with MBB. Compare with Figure 2. The nuclear margins are considerably more distinct, and several nucleoli are apparent. TCA-MBB stain. × 1300.

Fig. 4. Six hours after inoculation. The cytoplasm does not stain, but nucleoli appear more dense than in normal tissue. In some nuclei, bits of chromatin intermingled with very pale-staining substance may be noted. There is margination of chromatin, although typical inclusions are not evident. AFG stain. × 950.

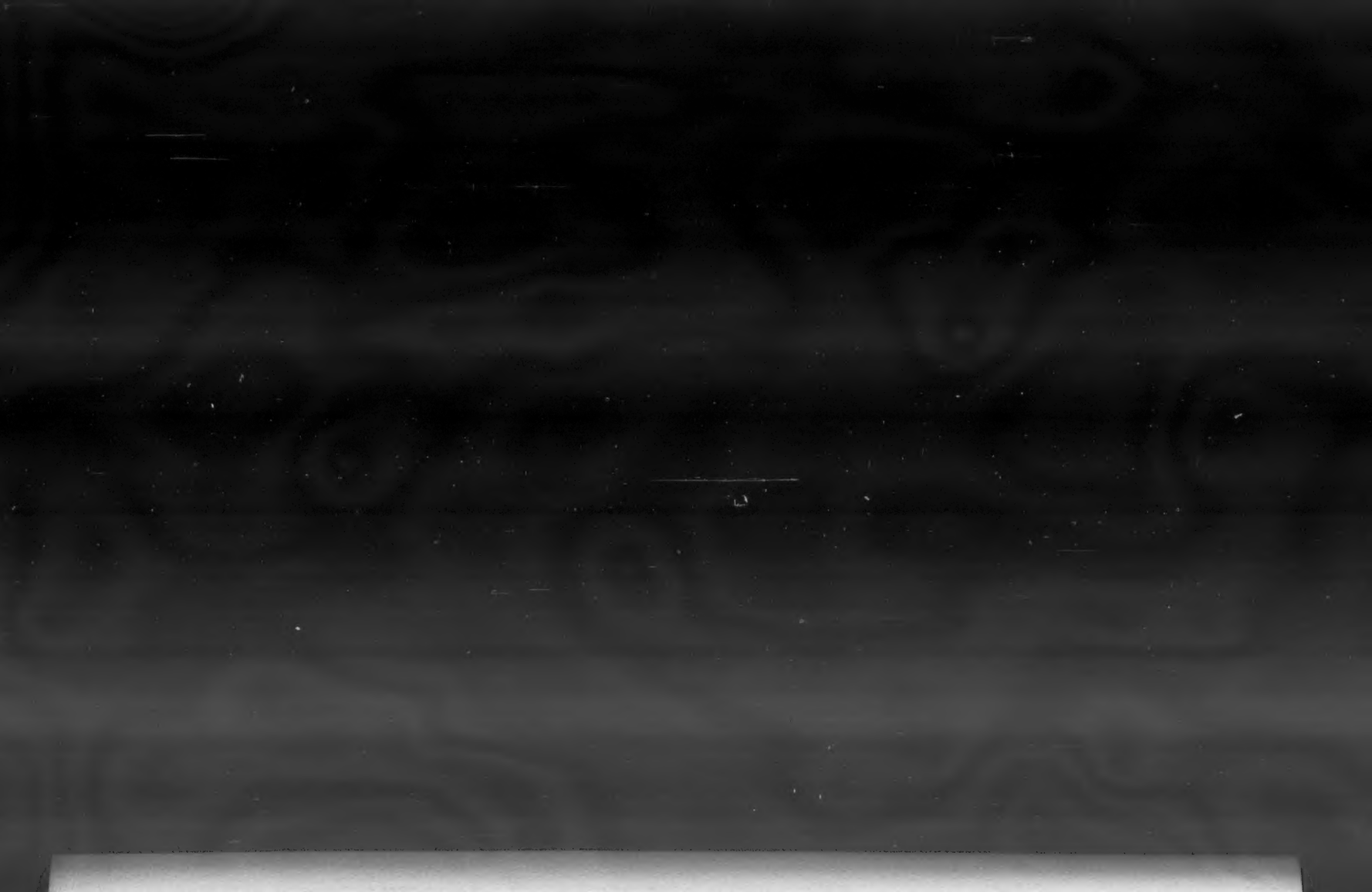
Fig. 5. Six hours after inoculation. The content of several nuclei is retracted and may be distinguished primarily because of the retraction and not because of staining difference. MBB stain. × 1200.

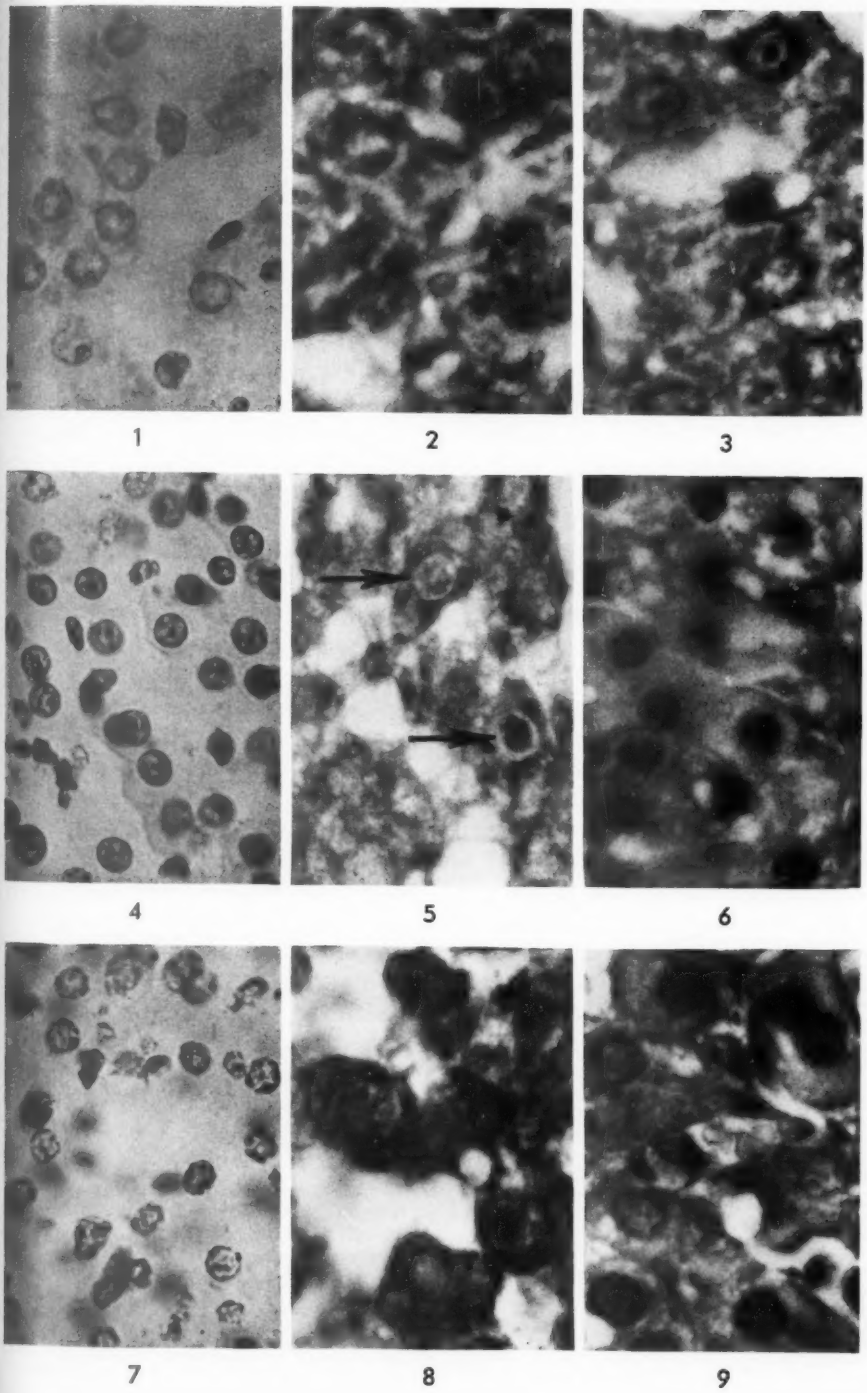
Fig. 6. Six hours after inoculation. The nuclei are quite dense. They contrast well with the much lighter cytoplasm, as well as with the unextracted nuclei shown in Figure 5. TCA-MBB stain. × 1300.

Fig. 7. Fifteen hours after inoculation. Cytoplasm is not stained. Nucleoli are no longer discernible and chromatin is arranged in beads about the periphery of the nuclei. Irregular pale-staining intranuclear networks are conspicuous, although inclusions as such are not recognizable. AFG stain. × 950.

Fig. 8. Twelve hours after inoculation. Cytoplasm is generally darker than at 6 hours and has a foamy appearance. The nuclei are dense and are difficult to distinguish from the dark cytoplasm. MBB stain. × 1300.

Fig. 9. Twelve hours after inoculation. Nuclei are stained less uniformly than at 6 hours, but still contrast distinctly with the much lighter cytoplasm. Some nuclear detail may be seen, particularly clumping of protein around the margin. Inclusions are in evidence. TCA-MBB stain. × 1300.





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THE HISTOLOGIC DISTRIBUTION OF RENIN IN THE KIDNEYS OF THE RAT AND RABBIT

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There is considerable evidence that the development of hypertension in experimental animals is related to the presence in the blood stream of a pressor polypeptide, angiotensin. This presumably arises as a consequence of the proteolytic action of a nephrogenic enzyme, renin, upon a plasma globulin, hypertensinogen.[1] Although renin has been shown to be present in the normal renal cortex of mammals and in marine life,[2] the cellular elements responsible for its formation have not been identified. Knowledge of the site of synthesis is essential to a study of the rate of production and release of this enzyme, and would assist materially in properly evaluating the importance of this factor in the genesis of hypertension, experimental and human.

In the present investigation, two general methods of approach were used. Animals in one group were given tartaric acid, an agent reported to destroy proximal tubular epithelium selectively, and the kidneys were then assayed for renin content. In a second group, the blood flow through the kidney was reduced to a degree where glomerular filtration ceased and various cortical structures underwent atrophy. Measurements of renin content were made in the kidneys of such animals and correlated with the structural alterations observed.

Renin synthesis has been attributed to different cortical structures by various investigators. Friedman and Kaplan,[2] using rabbits treated with sodium tartrate, implicated the proximal convoluted tubules. Taquini,[3] and Selye,[4,5] on the basis of several different techniques, came to the same conclusion. Yoshimura and Negishi[6] were unable to reproduce the results of Friedman and Kaplan and concluded that renin must be produced in some cortical structure other than the proximal convoluted tubule. Goormaghtigh[7] believed the liberation of a pressor substance by ischemic kidneys was related to the presence of hypertrophied granular cells (the so-called juxtaglomerular cells) in the wall of the afferent arterioles. Recent work by the Hartrofts,[8] and Tobian, Thompson,

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Twedt and Janecek[9] supports this hypothesis. The present studies in the rat and rabbit suggest that there is a close correlation between renin content and the state of the granular juxtaglomerular cells.

Material and Methods

Animals

Three groups of experiments were carried out. In group I, 33 Carworth Farm Nelson rats of both sexes, weighing approximately 200 gm., were divided as listed in Table I in order to establish the optimum conditions for producing selective tubular necrosis with sodium tartrate. They were sacrificed at various intervals to determine the time of appearance of this lesion. In group II, 15 rabbits, weighing between 1 and 1.5 kg., were similarly treated with sodium tartrate to evaluate species differences.

In a second approach to the problem, during a period of several months 9 hypertensive rats were collected from our colony under the following circumstances. Necropsies in 5 hypertensive animals that had died 48 hours following a contralateral nephrectomy revealed that the remaining "ligated" kidney was markedly shrunken and atrophic. Four additional hypertensive animals of this type were sacrificed and found to have atrophic "ligated" kidneys with concomitant hypertrophy of the contralateral kidney. Hypertension had been induced by tying a silk ligature about the left renal artery, with a #22 needle stylet placed alongside it, followed by prompt removal of the stylet. Blood pressures were measured by the microphonic method of Friedman and Freed[10] in rats under light ether anesthesia. These 9 rats are listed in Table III.

Preparation of Tartaric Acid

Racemic tartaric acid was partially neutralized to pH's ranging from 5.5 and 7.7 with sodium carbonate for the rat experiments. In the rabbit experiments, the pH was regularly 7.1. The grams of acid listed in the tables refer to the weight of the acid, not to the neutralized sodium salt. Subcutaneous injections at multiple sites were given following a period of fasting and dehydration.

Preparation and Bio-assay of Renin

All animals were sacrificed when terminally ill to avoid postmortem alterations, and the kidneys were removed promptly. A portion was minced and homogenized in the cold with 3.5 ml. of saline per gm. of tissue. The homogenates were agitated for 45 minutes in the cold, and then centrifuged at 30,000 × G for 45 minutes at 0° C. The resulting supernate (0.3 ml.) was injected via a catheter into the femoral veins of normal rats anesthetized with sodium pentobarbital, 3.5 mg. per hundred gm. of body weight. Blood pressure was measured directly, by means of a carotid artery cannula connected to a mercury manometer.

In Tables I, II, and III renin content is expressed in rat units per gm. of whole kidney. A rat unit of renin is arbitrarily defined as the amount of renal pressor substance required to raise the blood pressure of the test rat by 35 mm. of Hg. For example, 0.3 ml. of an extract of normal rat or rabbit kidney was found to raise blood pressure by 35 to 70 mm. of Hg and therefore is listed as containing 1 to 2 rat units of renin. The same volume, 0.3 ml., was always used in testing the extracts from the different groups. This amount of supernate, 0.3 ml., contained the renin from 0.069 gm. of whole kidney (cortex and medulla). By interpolation then, each gram of whole kidney from normal rats and rabbits contained 14 to 28 rat units of renin. Each extract was assayed in a normal rat which had received no previous injection other than the anesthetic agent. The kidneys of normal rats and rabbits contained on the average 19 rat units of renin per gm.

Preparation of Histologic Sections

Kidneys were fixed in Helly's fluid (Zenker's solution plus 5 per cent formalin). In addition to conventional hematoxylin and eosin staining, the Hartroft method for studying granular juxtaglomerular (j.g.) cells was used. The juxtaglomerular index (J.G.I.), a measure of the number and granularity of these cells, was also calculated.[7] In normal rats the mean J.G.I. was found to be 38, with a range of 15 to 48.

Observations

Rats Treated with Sodium Tartrate

The results are summarized in Table I.

Microscopic Observations. The lesion, when present, consistently involved all of the proximal convoluted tubules examined. Although no intact proximal tubules were seen, the glomeruli, j.g. cells, blood vessels

Table I

RATS TREATED WITH SODIUM TARTRATE

Group	No. of rats	Acid (gm./kg.)	pH	Fasted *	Sacrificed	Selective necrosis	Renin content †	J.G.I. ‡
3-B	3	none		72 hr.	After 72 hr.	No	17 ± 3	26 ± 3
4	6	none		5 days	After 5 days	No	16 ± 2	34 ± 14
1	5	2	5.5	No	48 hr. P.I.§	No	19 ± 5	39 ± 8
2	3	1	5.5	No	48 hr. P.I.	No	17 ± 3	39 ± 7
3-A	3	2.5	5.6	48 hr.	24 hr. P.I.	3/3	16 ± 2	36 ± 7
5	3	2	7.7	48 hr.	48 hr. P.I.	2/3	17 ± 3	32 ± 11
6-A	5	3	5.5	48 hr.	Serially‖	No	17 ± 3	26 ± 11
6-B	5	2.7	5.5	48 hr.	Seriallyπ	2/5#	18 ± 3	31 ± 11

* Injections given following the period of fasting and dehydration.

† Renin content is expressed in units per gm. of kidney. The median value and the deviation within each group are given.

‡ J.G.I. = juxtaglomerular index. The median value and the deviation within each group are given.

§ P.I. = post injection.

‖ Group 6-A sacrificed serially every 30 minutes, starting immediately after the injection.

π Group 6-B sacrificed serially every 30 minutes starting 2½ hours after the injection.

The last 2 rats sacrificed in group 6-B showed the "tartrate" lesion.

and distal convoluted tubules (including the macula densa) were unaffected (Fig. 1). The granularity of the j.g. cells was within normal limits. The lesion consisted basically of an extensive hydropic degeneration of the epithelium which progressed to frank cellular dissolution. Groups 6-A and 6-B received large doses of acid and were killed shortly thereafter. Bright yellow granules were consistently seen in blood vessel lumens, Bowman's spaces, and tubular lumens in these two groups of rats. The deposits presumably represented precipitated tartaric acid or a by-product. They did not resemble lipochrome or blood pigments and failed to take an iron stain.

Renin Content. Extracts of kidneys with selective tubular necrosis

contained as much renin as extracts of kidneys from untreated controls and kidneys that had failed to develop the "tartrate" lesion. The data in Table I reveal no significant differences in the number of renin units among the various groups.

Optimum Conditions for Producing the "Tartrate" Lesion in Rats. It was found best to fast and dehydrate the rats before and after the injection. This type of pretreatment apparently served to concentrate the acid in the proximal convoluted tubular epithelium. The optimum dose was between 2 and 2.5 gm. per kg. of body weight, at a pH of 7. Larger doses killed the animal, probably by acidosis, before histologic alterations developed. Following a moderately large dose, 2.5 gm. per kg., the lesion appeared within 4½ to 5 hours after injection.

Rabbits Treated with Sodium Tartrate

The results are summarized in Table II.

Microscopic Observations. The lesion in the rat, as detailed in the preceding section, was reproduced in the rabbit. Granular j.g. cells were unaffected and were equally prominent in the rabbit kidney. The calculated J.G.I. was similar in both species.

TABLE II

RABBITS TREATED WITH SODIUM TARTRATE

Rabbit no.	Acid * (gm./kg.)	Fasted (hr.)	Appearance post injection †	Selective necrosis	J.G.I.	Renin ‡
206	1.7	48	Listless 36 hr.	Yes	48	18
207	1.7	48	Listless 36 hr.	Yes	44	21
208	1.7	48	Listless 36 hr.	Yes	53	14
209	1.7	48	Listless 36 hr.	Yes	24	18
210	1.7	48	Listless 36 hr.	Yes	29	14
211	1.7	48	Frisky 36 hr.	No	28	21
212	1.5	48	Listless 36 hr.	Yes	48	15
213	1.5	48	Frisky 36 hr.	Yes	28	14
214	1.5	48	Listless 36 hr.	Yes	22	18
215	1.5	48	Listless 36 hr.	Yes	29	14
216	1.5	48	Listless 36 hr.	Yes	42	15
217	1.5	48	Listless 36 hr.	Yes	29	14
218	None	48	Frisky 36 hr.	No	39	14
219	None	No	Frisky 36 hr.	No	48	14
220	None	No	Frisky 36 hr.	No	42	14

* Injections given after the period of complete fasting. Food and water given *ad libitum* after injection.

† All animals sacrificed 36 hours after injection.

‡ Renin content is expressed in rat units of renin per gm. of rabbit kidney.

Renin Content. Extracts of rabbit kidneys with the "tartrate" lesion contained equivalent amounts of renin when compared with kidneys of controls or kidneys without the "tartrate" lesion.

Hypertensive Rats with Atrophic, Ligated Kidneys

The results are summarized in Table III.

Microscopic Observations. Hematoxylin and eosin-stained sections of the shrunken "ligated" kidneys revealed extensive atrophy of all renal tubules. Both cortical and medullary tubules were greatly diminished in number. Hyaline droplet degeneration was marked. The glomeruli, which were well preserved, and the arteries and arterioles stood out prominently amid the degenerated tubules which could be recognized only as diminutive rings and clumps of small cells (Figs. 2 and 3). Although most of the damaged tubules showed no evidence of lumens, in a few instances it was possible to distinguish markedly flattened epithelial cells lining tubular lumens in the cortex. The tubular atrophy was associated with a crowding of the glomeruli and blood vessels. In addition, there was marked interstitial fibrosis (Fig. 4). The special portion of the distal convoluted tubule known as the macula densa could no longer be identified. Normally these cells are large and columnar and lie adjacent to the glomerular vascular pole. Such cells were not seen. Aggregates of acute or chronic inflammatory cells were not present in any of the sections.

TABLE III

HYPERTENSIVE RATS

Rat no.	Blood pressure (mm. of Hg)	Duration of hypertension	L.K.*		C.K. †	
			J.G.I. ‡	Renin §	J.G.I. ‡	Renin §
39	175	4 weeks	60	42	0	0
40-A	170	4 weeks	58	36	6	0
40	180	12 months	83	47	3	0
68	200	12 months	129	60	0	0
29	170	3 weeks	59		6	0
25	190	6 months	115		0	0
26	200	6 months	98		0	
36	185	10 months	118		1	
74	190	11 months	105		1	0

* L.K. = ligated, atrophied kidney.

† C.K. = contralateral, hypertrophied kidney.

‡ The mean juxtaglomerular index in normal rats is 38, with a normal range of 15 to 48.

§ The average renin content of normal rat kidneys is 19 units per gm., with a range of 14 to 28 units per gm.

By using the special staining technique of Hartroft, it was possible to show an absolute increase in the number and the granularity of juxtaglomerular cells associated with each glomerulus (Figs. 5 and 6). The J.G.I. was 2 to 3 times that of normal animals. Some of the kidneys were the seat of partial infarction, but had distinctly viable portions of

cortex which showed the changes described above. The untouched contralateral kidneys were hypertrophied and showed moderate degrees of arterial and arteriolar sclerosis. Their juxtaglomerular cells appeared devoid of granules.

Renin Content. Assays of the renin content of 4 "ligated" kidneys showed a 2- to 3-fold increase in activity. These extracts produced blood pressure elevations in the range of 90 to 150 mm. of Hg in test rats. Extracts of the contralateral, hypertrophied kidneys were inactive and did not elicit a measurable pressor response.

Discussion

The foregoing observations led us to conclude that the granular juxtaglomerular cells residing in the walls of afferent arterioles are most likely responsible for synthesizing and storing renin. The other structures in the cortex, i.e., glomerular tufts, proximal and distal convoluted tubules, and collecting ducts could be excluded on the following grounds: The proximal convoluted tubules were unequivocally eliminated by the tartaric acid experiments in rats and rabbits. Complete destruction of these tubules did not lower the renin content. The distal convoluted tubules, including the portion known as the macula densa, and the cortical collecting tubules were ruled out as sites of renin production on the basis of the observations in the atrophic, "ligated" kidneys in hypertensive rats. The atrophy selectively involved all of the tubules, leaving only partially fibrosed, diminutive masses of collapsed tubular epithelium. These were composed of small cells separating glomeruli and increased numbers of hypergranulated j.g. cells. The glomeruli, being essentially vascular structures, did not undergo atrophy. Such kidneys contained up to 3 times the normal quantity of renin.

It is generally agreed that active or increased secretion in endocrine or exocrine structures is accompanied by morphologic changes in the secreting cells. This is manifested either by hyperplasia, by an increase in the size of each cell, or by an accumulation of cytoplasmic granules. The renal tubules, including special portions such as the macula densa, showed signs of severe atrophy. Since the glomerular tufts remained intact, they could not be excluded with certainty. The granular j.g. cells showed hyperplasia, an increase in cell size, and a marked accumulation of cytoplasmic granules, and thus appeared to be the most likely sites of renin production. Further corroborative evidence was provided by the fact that in instances where the j.g. cells were depleted of granules and involuted, as in the hypertrophied contralateral kidney, the renin content, as determined by bio-assay, dropped correspondingly below measurable limits.

Additional support for the j.g. concept is provided by the fact that "ligated" kidneys not only can be shown to have increased renin content, but to be releasing increased quantities into the blood stream. Quinby, Dexter, Sandmeyer and Haynes[11] have detected increased levels of renin in the renal venous blood from kidneys in dogs in which the renal artery was temporarily occluded. Page[12] found increased amounts of renin in the renal vein blood of dogs made hypertensive by cellophane or silk perinephritis, or by clamping the renal artery. Haynes and Dexter[13] found rising levels of renin in the plasma of dogs during the development of experimental renal hypertension. Thus, consideration of both morphologic and physiologic observations strongly suggests that there is an "increased secretion" of renin from "ligated" kidneys.

Our findings and conclusions are essentially in agreement with those of Yoshimura,[6] Hartroft,[8] and Tobian[9] and their co-workers. The latter two teams in separate investigations showed a good correlation between renin levels and the J.G.I. in various types of kidneys. Recently, however, Nairn, Fraser and Chadwick,[14] using fluorescent-labeled "antirenin," concluded that renin is probably located in glomerular tufts. The inherent difficulty with this approach, as Nairn points out, stems from the fact that the antigen used, renin, has not been completely purified, and the resulting antibodies are mixed and not necessarily specific for renin. The investigations of Bing and associates[15,16] do not support Nairn's conclusion. By microdissection of freshly cut thin kidney sections, they found that cortical slices devoid of glomerular tufts had normal quantities of renin. Furthermore, a marked drop in renin content was observed only in preparations where the structures adjacent to the glomeruli had been destroyed. In their most recent publication, they were unable to demonstrate renin in glomerular tufts, which had been carefully dissected, and then extracted for renin. They suggested that renin was produced by portions of the distal convoluted tubules, such as the macula densa, or the j.g. cells.

Hess and Pearse[17] have recently concluded that the macula densa of "ligated" kidneys was involved because they were able to demonstrate in these cells an increase in the cellular enzymes usually associated with anabolic processes. The severe atrophy affecting the macula densa in the present investigation does not favor such an interpretation.

Summary

In an attempt to localize renin, its content was correlated with the number and granularity of the juxtaglomerular cells in 3 types of kidneys.

In the first type, although the proximal convoluted tubules were completely destroyed with sodium tartrate, the renin content and the J.G.I. remained within the normal range, indicating that renin is not produced in the proximal convoluted tubules.

In the second type, the arterial circulation was curtailed by a constricting ligature to a point where all cortical and medullary tubules atrophied greatly, while the glomeruli were relatively unaffected; granular j.g. cells showed marked hyperplasia and increased granularity. Such kidneys contained up to 3 times the normal quantity of renin, suggesting that the hyperplastic j.g. cells were the most likely sites of renin formation.

The third type of kidney studied was the untouched but hypertrophied kidney in hypertensive rats. These kidneys contained no renin and displayed involuted, agranular j.g. cells. This, again, supported the concept of a direct correlation between renin content and the J.G.I.

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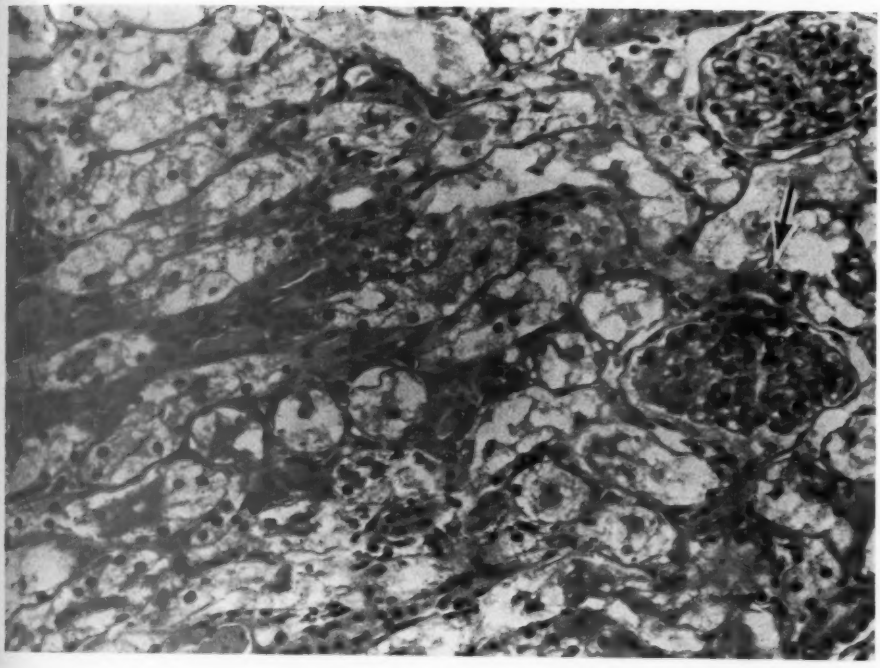
[*Illustrations follow*]

Legends for Figures

FIG. 1. Renal cortex in a rat with a "tartrate" lesion. There is extensive degeneration and dissolution of cells lining the proximal convoluted tubules. The macula densa (arrow) is visible at the vascular pole of one glomerulus. Hematoxylin and eosin stain. × 150.

FIG. 2. Kidneys in a hypertensive rat (4 weeks' duration), cut in midsagittal section. The "ligated" kidney is shrunken and atrophic; the contralateral kidney is hypertrophied. Hematoxylin and eosin stain. × 2.

FIG. 3. Area in the atrophied "ligated" kidney in Figure 2, showing extensive tubular atrophy and crowding of glomeruli. Hematoxylin and eosin stain. × 150.



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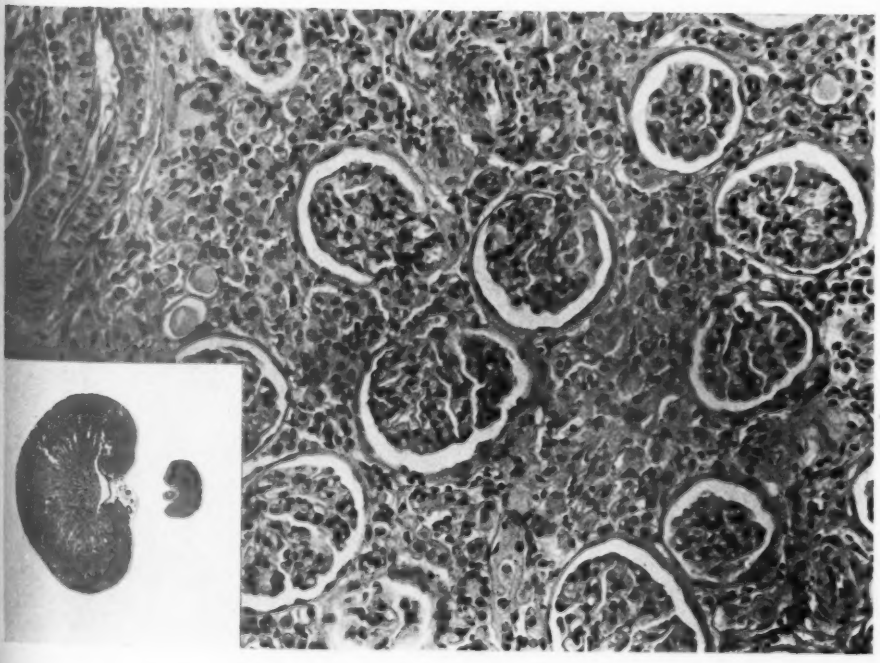

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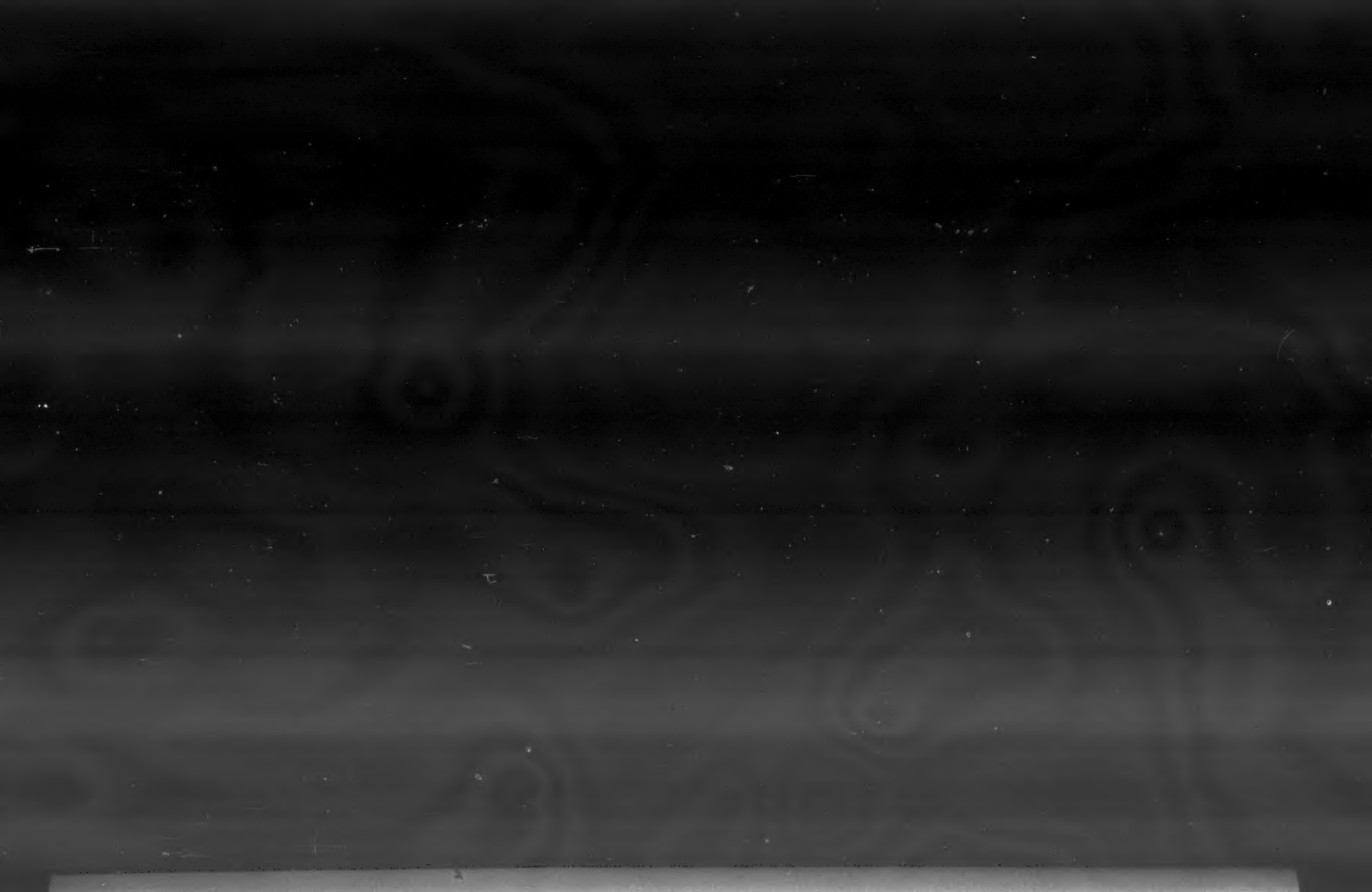
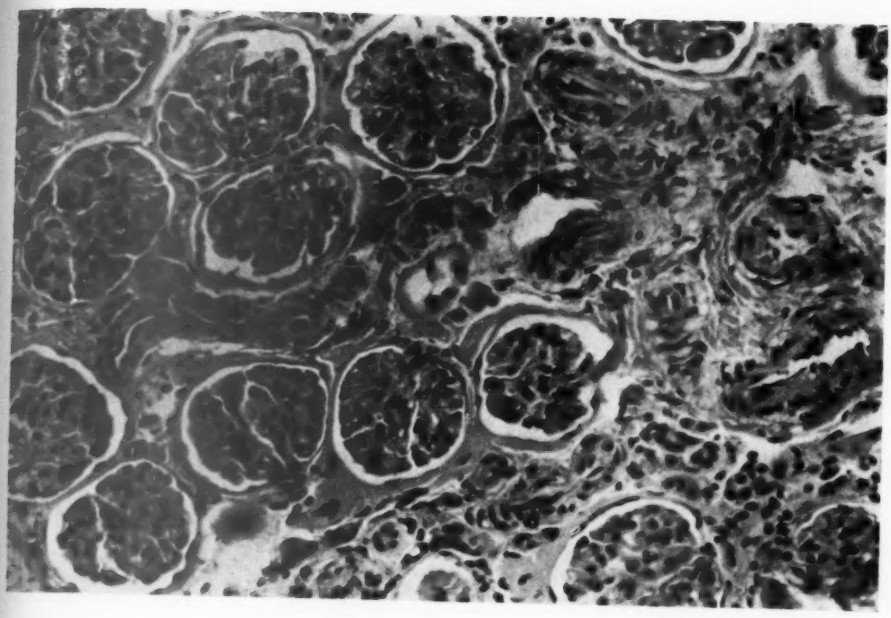
FIG. 4. Atrophied "ligated" kidney in a rat with hypertension of 12 months' duration. There is marked interstitial fibrosis and tubular atrophy. The glomeruli and blood vessels are well preserved. No inflammatory cells are present. Hematoxylin and eosin stain. × 180.

FIG. 5. A representative cluster of granular j.g. cells in an atrophic "ligated" kidney stained by the method of Hartroft. The cells are increased in number and size and are hypergranulated (compare with Fig. 6). Bowie stain. × 1250.

FIG. 6. Section of normal rat kidney showing a representative aggregation of granular j.g. cells. Bowie stain. × 1250.





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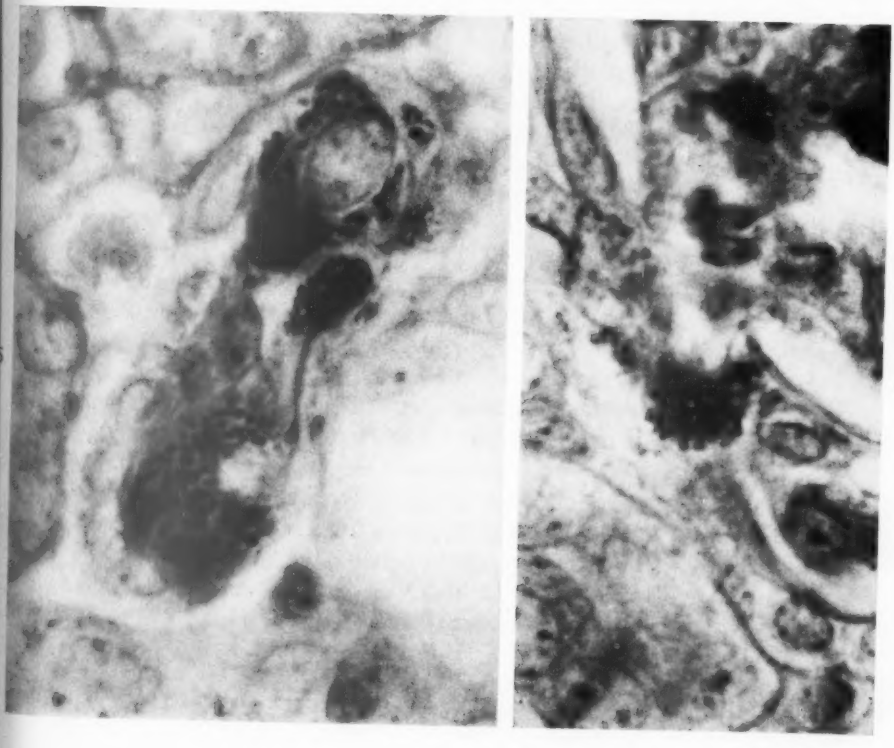

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SIDEROSIS ASSOCIATED WITH INCREASED LIPOFUSCINS AND MAST CELLS IN AGING MICE

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Recent studies of the histochemical properties of mucopolysaccharides have demonstrated variability in the staining reactions of mast cells.[1] In general, the sulfated mucins in these cells, like those in cartilage and certain glands and goblet cells, fail to stain with Alcian blue despite a strong affinity for azure A and safranin. Lack of Alcian blue staining correlates with an abundance of strongly acid sulfate esters in naturally, as well as artificially, sulfated mucins. Some mast cells in the mouse, however, react with Alcian blue, so that with a combined Alcian blue-safranin stain, certain of the cells color blue and others red. Azure A alone stains both types purple, but the Alcian blue-positive cells appear to be smaller and less strongly metachromatic. Such variability may have physiologic implications, possibly indicating functional depletion of sulfated mucopolysaccharides. Thus it appeared of interest to investigate the distribution in mouse tissues of the two types of mast cells demonstrable by the Alcian blue-safranin staining sequence.

Differences in rat mast cells have been described previously by Riley, who distinguished a class I type of cell located in the adventitia of muscular vessels and showing dense agranular orthochromatic cytoplasm, from a class II type located near distal capillaries and containing abundant metachromatic granules.[2] Although with the Alcian blue-safranin procedure occasional Alcian blue-stained cells have been encountered in arterial walls, a definite correlation of distribution with that of Riley type I cells has not been possible in the mice thus far examined. A different kind of distribution was observed, however; the Alcian blue-staining mast cells were commonly found in older mice in close proximity to large phagocytes filled with granular, yellow-brown pigment indicative of hemosiderin or lipofuscin. Accordingly, the possibility has been considered that a relation may exist between the location of mast cells and the distribution of stainable iron and fat stained by the Ciaccio method. The occurrence of hemosiderin in the spleen and lymph nodes[3] and of cytosiderin in the mammary gland[4] has been described. There

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are also reports of lipofuscin in the thymus,[5] adrenal,[6] and testis.[7] However, the distribution of these two materials in normal mice, as in the case of lipofuscin in humans [8–11] has not been determined. Mouse tissues have therefore been examined for the 3 components. This required comparison of young and aged mice because it was soon evident that in some areas there were fewer mast cells in young than in aged mice, and that tissues from the younger group lacked the stainable iron and lipid material so prevalent in the older animals. Two main observations emerged from this investigation: (1) Year-old, in contrast to month-old, mice exhibited large amounts of iron and lipofuscin in closely associated phagocytes throughout the body, as well as within the cytoplasm of some parenchymal cells. (2) Older mice, in contrast with young animals, showed numerous mast cells intimately associated with the iron or fat-laden phagocytes. The cells situated in relation to the heaviest concentrations of siderotic or lipid substance contained weakly metachromatic and Alcian blue-reactive cytoplasm.

Methods

Tissues from 1-month-old mice and 10 to 14-months-old, retired breeder mice were fixed 24 hours at 25° C. in neutral calcium acetate formalin prior to paraffin embedding.

An equal number of male and female mice from the C_3H/p and NIH colonies maintained by brother-sister mating as well as mice from the NIH general purpose, randomly bred colony were examined. A series of 2 to 6 young and 6 to 12 aged animals in each strain was examined to obtain the average picture presented for each organ. There was only moderate variation between individuals of one strain or between strains, but certain sex differences were noted.

In order to demonstrate mast cells, sections were stained 30 minutes in 0.02 per cent azure A in N HCl (pH 0.5) or 1 minute in 0.5 per cent safranin at pH 1.5. Alternatively a 20-minute exposure to Alcian blue in 0.3 per cent concentration in 3 per cent acetic acid was followed by the safranin or azure A in pH 1.5 buffer. Perls's ferrocyanide method [12] was used to visualize ferric iron in the tissues, by employing 1 per cent ferrocyanide in 0.12 N HCl at 60° C. for 30 minutes. As a means of directly evaluating the relationship between siderophages and mast cells, sections were stained with safranin at pH 1.5 after the ferrocyanide procedure for iron. Sudan black B, spirit blue, Mallory's hemofuscin procedure, the ferric chloride ferricyanide reduction test, the acid-fast staining procedure and the periodic acid-Schiff (PAS) and peracetic acid-Schiff (PAAS) methods were used to demonstrate lipofuscin as detailed by Lillie [11]; Lillie's Nile blue sulfuric acid technique was also utilized.[13] Sudan black B, Nile blue, and spirit blue demonstrated the Ciaccio type lipids comparably. All the stains were employed in each organ. Results with the peracetic acid-Schiff and acid-fast methods are not recorded since, in general, they were weaker than the other methods. Values in the tables indicate microscopic estimation of the abundance of cells or of stainable substance.

Halmi's aldehyde fuchsin stain [14] was stored 24 hours after preparation and then used following one hour oxidation of the section in freshly prepared peracetic acid.[11] This peracetic acid-aldehyde fuchsin (PAAF) procedure, used for special purposes by Landing, Hall and West [15] and Fullmer,[16] was first employed in this laboratory in an investigation of the mechanism of mucus staining by aldehyde fuchsin. The pro-

cedure was incidentally found to color thymic, adrenal and ovarian lipofuscins a dark purple and to stain particles in parenchymal cells of aged mice. It is introduced here for this reason. Aldehyde fuchsin without prior oxidation and Mallory's basic fuchsin stain for hemofuscin failed to demonstrate parenchymal cytoplasmic granules. The fluorescence of lipofuscins described by Hamperl [17] was investigated by means of a Leitz high pressure 150 watt mercury arc lamp as a source of 3,600 and 4,150 Å emission. This was used in conjunction with a Leitz BG 12, 8 mm. blue lamp filter and an OG 1, 2.5 mm. orange ocular filter. Sections to be examined for fluorescence were deparaffinized and mounted unstained in cellulose caprate.

Results

Year-Old Mice

Mast cells infiltrate the lamina propria or stroma of many organs throughout the body, frequently lying in close association with comparable numbers of siderophages and lipophages (Table I and Figs. 1 to 3, 6 to 10, 12, 13 and 18). Epithelium in many organs reveals cytoplasmic granules or globules of PAAF stainable material and in some instances of cytosiderin as well (Table I and Figs. 3, 11 and 14 to 17).

Roughly half of the mast cells in the medulla of cervical lymph nodes show relatively weak metachromatic staining with azure A or safranin. It is assumed that these are the same cells as those present in the same location and in comparable numbers staining blue or purple with the Alcian blue-safranin sequence (Fig. 1a). The remaining cells, especially those in the cords, color red with the latter procedure and dark purple with azure A. Extracellular metachromatic granules and fibrils occasionally surround mast cells in the sinuses. The numerous mast cells in the hilar stroma are larger, strongly metachromatic, and color red with the Alcian blue-safranin procedure. Some variability in cervical lymph node mast cell content has been encountered, but it has not as yet been determined whether the differences represent a constant characteristic. Peri-aortic, peripancreatic and mesenteric lymph nodes in general contain relatively few mast cells, siderophages or lipophages. Mast cells and siderophages have been noted in lymph nodes of mice by Dunn who observed increased numbers of such cells in the nodal hili in aged mice.[8]

Unlike some of the strains of mice investigated by Deringer and Dunn,[18] the present group lack mast cells in the spleen. Splenic siderophages are abundant in all strains.

Iron-laden phagocytes in submaxillary glands are much more numerous in male than in female mice. This distinct sex difference suggests a possible derivation of the iron from the terminal serous tubules which are more prominent in males.[19] Granules of cytosiderin are so abundant in the cytoplasm of parotid intercalated ductal epithelium that the Prussian blue procedure selectively stains this segment of the gland (Fig. 3). Minute PAAF-positive and yellow fluorescent granules are also evident

TABLE I

RELATIVE PREVALENCE OF STAINABLE IRON, LIPOFUSCIN AND MAST CELLS OF YOUNG AND AGED MICE

Tissue	Year-old mice					Month-old mice		
	Parenchymal cytosiderin	Siderophages	Parenchymal or epithelial PAAF-reactive particles *	Lipophages	Mast cells	Stainable iron	PAAF or Nile blue reactive material	Mast cells
Cervical lymph nodes, sinuses		++++		+++	++++	o	o	±
Spleen		++++		++++	o	+	±	o
Submaxillary gland	o	+++†	o	+	++	o	o	+
Parotid gland	++	+++	o−+	++	++	o	o	+
Sublingual gland	o	++	o	+	+	o	o	+
Exorbital lacrimal gland	o	++	o	++	++	o	o	+
Larynx and trachea	o	+	o		+	o	o	±
Lung	o	+	o	▫	±	o	o	o
Tongue: fibrous septums		++			+++	±	o	++
Esophagus	o	±	o	o	+	o	o	o
Stomach	o	+	o	+	+++‡	o	o	+++‡
Duodenum	++	+	o	+	+	++	o	+
Colon	o	+++	o	+	++	o	o	+
Liver	o−+	o−++	o−+	o−++	±	o−+	o	o
Pancreas	o	±	o	±	±	o	o	o
Thymus		+§		++++	+§	o	o	o
Thyroid gland	o	+	o		+	o	o	o
Adrenal gland	o	+++	o	++++	o	o	o	o
Testis	o	+		+++	o	o	o	o
Ovary		+++		+++	+			
Kidney	o−+	o−++‖	o	±	±	o	o	o
Urinary bladder	o	±	o		±	o	o	o
Fallopian tube	o	+++	++	++	+++	o	o	±
Myometrium	o	++++	o	++++	++++	o	o	±

Endometrium	o	++π	o−++	++	±π	o	o	o
Vagina	o	++#	o	±	++	o	o	±
Ductus epididymis	o	++	+++	++	+	o	o	±
Ductus deferens	o	++	++	++	++	o	o	±
Seminal vesicle	+++	++	+++	++	++	o	o	±
Prostate gland	o	+	+++	+	++	o	o	±
Coagulating gland	o	+	++	+	++	o	o	±
Skin: corium	o	++	o		+++	o	o	+++
Female mammary gland	++++	++	+++**	++	+++	o	o	±
Preputial gland	o	+++	o	+	+++	o	o	+
Central nervous system	o−+	+	+++	++	o	o	o	o
Peripheral nerves and ganglia		++		+	++	o	o	++
Bone marrow		++		++	±	+	±	±
Cardiac and skeletal muscle	o	+	o		+	o	o	±
Smooth muscle	o		o			o	o	

* PAAF: peracetic acid-aldehyde fuchsin stain.
† Male, +++; female, ++.
‡ Abundant in the muscularis at the junction of the glandular and nonglandular portions, and in the superficial epithelium.
§ In the lining capsule and occasionally infiltrating along larger vessels in the cortex.
|| Male, ±; female, ++.
π Plasma cells and eosinophils are often numerous.
Frequent in lamina propria at 2 to 3 months of age.
** Large aggregates of sudanophilic material are present in ex-breeder mice. These are less prominent in aged unbred females.

in the same epithelium. Darkly staining mast cells and siderophages densely populate the fibrous septums and perivascular spaces in the areolar tissues at the hili of salivary glands (Figs. 4 and 5).

At all ages the epithelium in the proximal duodenum contains the cytosiderin granules observed originally by Macallum and presumed to occur in association with the absorption of dietary iron.[20,21]

In the liver, mast cells, as a rule, are scarce, but the amount of stainable iron and lipofuscin varies. About half of the older animals (with extensive siderosis elsewhere) show few or no siderophages, and exhibit no epithelial iron deposit. In some mice a faint diffuse Perls reaction appears in parenchymal epithelium containing few or no distinct granules. A few to numerous iron-positive Kupffer cells and lipophages are seen in a number of animals, especially those 18 months old. The PAAF stain visualizes minute cytoplasmic particles in the hepatic epithelium in approximately half of the animals.

Foam cells are frequent in the cortex of the thymus. First demonstrated with fat stains by Loewenthal and Smith,[5] these react even more intensely with the PAAF procedure. The adrenal, testis and ovary contain few or no mast cells but reveal nests of strongly sudanophilic cells[6,7] with variable iron staining and PAAF reactivity (Table I). In an investigation of the basophilia of the ovarian lipofuscin, it has been observed that the reactivity toward 0.02 per cent azure A, although normally very weak below pH 3, is marked at pH 1.5 in sections which have previously been saponified 20 minutes in 1 per cent potassium hydroxide in 70 per cent ethanol.

The kidney and urinary bladder contain few mast cells, siderophages or lipophages except for a number of iron-positive interstitial cells at the junction of the outer and inner stripe in the outer zone of the medulla (Table I). In the NIH general purpose mice, at least, these siderophages have been observed only in females. Renal epithelium shows no stainable iron in about half of the year-old animals. Of the remainder, half reveal iron-positive granules in some of the proximal convoluted tubules; the others show only a diffuse faint green coloration.

Although mast cells have been described in the uterus of the mouse and changes during the estrous cycle have been investigated,[22] there has been no comment on their increase with age or their association with siderophages and lipophages to produce the extremely heavy infiltration occurring in all older female mice regardless of the cyclic phase. Like many of the mast cells in cervical lymph nodes, a large number in the inner muscular layer of the myometrium stain relatively weakly with azure A and appear blue or purple rather than red with the Alcian blue-safranin sequence (Fig. 1b). Fluorescent cytoplasmic globules often

visualized in the endometrial epithelium stain with Sudan black or peracetic acid-aldehyde fuchsin. The matter has not been investigated, but the inconstant appearance of intra-epithelial Ciaccio-positive lipid indicates that it may occur in relation to the estrous cycle. In virgin females, mast cells, siderophages and, to a lesser extent, lipophages are very numerous in the uterus; the density of the infiltrate, however, does not appear to equal that seen in the year-old retired breeder mice. Distribution differs also in that the virgin animals show a more diffuse infiltration without the focal, dense, almost fused aggregates of pigmented siderophages and lipophages and mast cells which presumably develop at implantation sites.

As shown in the tables and illustrations, the PAAF procedure reveals purple-staining cytoplasmic particles in the epithelium of most organs in the genital tract. More remarkable are the granular deposits of cytosiderin in the epithelium of the seminal vesicle (Fig. 15). An increase of mast cells, siderophages and lipophages with age is particularly noticeable in the stroma of these organs. In the wall of the ductus deferens many mast cells stain blue with the Alcian blue-safranin sequence.

Of the numerous mast cells in the skin, those at the tips of the papillas tend to stain blue with the Alcian blue-safranin procedure whereas others appear reddish purple to bright red. Mast cells in the labia are especially abundant and intermingle intimately with melanophores. Precise quantitative determinations properly controlling the varied factors which influence mast cell counts [23] would be needed in order to determine with certainty whether the numbers of dermal mast cells increase with age.

The epithelium in mammary ducts contains abundant cytosiderin; insoluble lipid and mast cells are intimately associated (Figs. 1f and 19). Olivi has demonstrated a variation in mast cells in the mouse mammary gland resulting from hormonal stimuli.[24] Stainable iron in the resting mammary glands of female mice was observed originally by Rawlinson and Hankinson.[4]

Although commonly encountered in peripheral nerves and ganglia, mast cells are rarely if ever found in the central nervous system. Perls's method stains neurons in isolated foci in the brain and also subpial phagocytes. Siderophages are common in peripheral nerves and ganglia. The siderotic neurons resemble the nonhemorrhagic iron-positive conglomerations observed by Strassmann in the globus pallidus of aged humans.[10] The PAAF procedure demonstrates cytoplasmic granules morphologically similar to the human lipofuscins [8,25] in most central neurons (including Purkinje cells) and in subpial phagocytes.

Month-Old Mice

Abundant mast cells infiltrate the tongue, stomach, skin and peripheral nerves in young mice (Table I), but cytosiderin (except in the duodenum[20,21]), siderophages, PAAF-positive cytoplasmic particles in parenchymal cells, and lipophages are essentially absent.

TABLE II

HISTOCHEMICAL REACTIVITY OF LIPOPHAGES AND EPITHELIAL PARTICLES IN AGED MICE

Site		PAAF	Fat stains *	PAS	Mallory hemofuscin	Ferric chloride-ferricyanide	Fluorescence
Lymph nodes and spleen	Phagocytes	++	+++	++	+	++	++
Exorbital lacrimal & salivary glands	Phagocytes	++	++		o		++
Parotid gland	Epithelium	+	o	o	o	o	+
Intestine	Phagocytes	++	++		o	++	++
Liver	Phagocytes	+++	+	+	±		
Liver	Epithelium	+	o	o	o	o	
Thymus	Phagocytes	++++	+++	++	+	+	++++
Adrenal & ovary	Phagocytes	++	+++	++	+		+++
Testis	Phagocytes	±	++++		+		+++
Fallopian tube	Epithelium	++	o	o−+	o	o	o
Uterus	Phagocytes	++	+++	++			+++
Ductus epididymis, Ductus deferens, Seminal vesicle, Prostate gland	Epithelium†	+++	o−+‡	o−+§	o	o	o−+‡§
Mammary gland	Epithelium	+	+++				+++
Central nervous system	Neurons	+++	o−+	o−+	o	o	

* Sudan black, Nile blue, or spirit blue stains.

† Infrequent dense bodies larger than those with definite cytoplasmic localization appear between epithelial cells, possibly in phagocytes. They stain darkly with PAAF and lightly with PAS and Nile blue methods.

‡ Negative in all these sites except for weak reactivity of the most intensely PAAF-positive particles in the epididymis.

§ Negative except for weak reactivity of a few of the PAAF-positive particles in the seminal vesicle.

Table II shows the correspondence between the results given by the PAAF method and those obtained with the stains which demonstrate lipofuscin in some areas. As notable exceptions, however, there are almost exclusive staining of cytoplasmic bodies in epithelium by the PAAF procedure and a weak PAAF staining of sudanophilic testicular lipofuscin. The heterogeneity of these substances is apparent in their varied reactions.

DISCUSSION

In older mice, iron- and lipid-laden phagocytes accumulate in the basement membrane region and stroma of most epithelial structures.

This might be explained by assuming that these cells phagocytize degenerating lipoproteins and iron-containing proteins such as cytochromes. These substances are extruded from viable cells in the turnover of cellular enzymes or escape terminally from epithelium in the process of replacement.

The marked siderosis of the uterus in mature virgins presumably results from cellular replacement during the estrous cycle and resorption of degenerating metestrous epithelium.[20] The heavier deposits encountered in ex-breeder animals apparently develop in connection with the disposal of placental remnants. The relative abundance of siderocytes in the lamina propria of the vagina and their early appearance in contrast to the esophagus correlates with the occurrence of cyclic changes in vaginal epithelium. The sex difference in siderosis of the submaxillary glands probably relates to sex differences in structure[19] and, undoubtedly, function and metabolism as well. The wandering macrophages presumably transport the siderotic substance to regional lymph nodes.

The presence of iron-containing granules in the epithelium of the seminal vesicles, parotid and breast in older animals is of interest in this connection. Little is known of the fate of "worn out" cytoplasmic proteins. However, the appearance of aggregates such as these could represent a phase in their discard. Physiologically active iron in hemoglobin, myoglobin and the cytochromes, bound in coordinate and covalent linkage to pyrrole and protein, fails to form Prussian blue with Perls's ferrocyanide. There is no apparent explanation for the selective deposition of iron in these organs.

A similar basis may be considered for the parenchymal cytoplasmic particles visualized in several organs with the PAAF stain. Sudan-stained macrophages and PAAF-positive phagocytes, often distinguishable from mast cells, populate the stroma adjacent to affected cells in organs containing these bodies. This suggests that the phagocytized material is derived from the parenchymal particles. The concurrent focal increase of both PAAF and ferrocyanide-reactive substance indicates a possible common source for both. The nature of the PAAF-stained material is somewhat uncertain in view of the lack of understanding of the histochemical significance of this staining method. In our experience, in addition to mucins and certain proteins, the stain demonstrates known sudanophilic material in the ovary, adrenal and thymus and in phagocytes. The intra-epithelial and neuronal particles are absent in young animals. For these reasons it is likely that the PAAF-positive intracytoplasmic particles in aged mice stain because of the presence of a Ciaccio type lipid and belong to the class of substances designated lipofuscins. The testis, seminal vesicle, vas deferens, epididymis and

brain are common sites for the deposition of lipofuscins in senile human subjects.[4,8] On the other hand, the parenchymal cytoplasmic bodies in aged mice differ from human lipofuscins in that they are not visibly pigmented, are absent from both smooth and cardiac muscle, and are not demonstrable by any of the common staining methods for lipofuscins except for a weak sudanophilia in the epididymis and a pale PAS reaction in the brain, fallopian tube and, occasionally, the seminal vesicle. Whether the singular capacity to demonstrate cytoplasmic particles with the PAAF stain in aged mice represents an unusual sensitivity in demonstrating lipofuscin or a reactivity with other previously undetected substances awaits further experience.

The intra-epithelial bodies, apparent in formalin-fixed tissue without chromate and silver impregnation or treatment with osmic acid, do not wholly conform to the conventional Golgi body despite some morphologic resemblance. However, in accord with the view of Kirkman and Severinghaus,[27] the Golgi apparatus might be visualized in unoxidized, formalin-fixed tissue if the dehydrated lipid content had been rendered insoluble through autoxidation or some other change. On the other hand, it appears possible that the cytoplasmic particles are related to the lysosomes as delineated by de Duve.[28]

The distribution of stainable iron in the year-old mice differs from that seen in hemolytic jaundice, repeated transfusions with citrated blood, or prolonged iron therapy.[29–35] In particular the inconspicuous or negligible involvement of the epithelium and Kupffer cells in the liver and the epithelium of the kidney and pancreas on the one hand, and the presence of stainable iron in the epithelium of seminal vesicles, terminal parotid tubules and mammary glands, and the relative abundance of iron in cervical lymph nodes, on the other, distinguish the siderosis in aged mice from other forms of hemosiderosis. That the iron demonstrated here is not a consequence of a hemolytic process is further indicated by the absence of anemia, reticulocytosis, or erythroid hyperplasia in the bone marrow in the aged mice with siderosis. Blood smears obtained before and after splenectomy failed to reveal erythrocytic parasites such as hemobartonella, Eperythrozoon, or Grahamella.

Since the aged mice lack siderotic deposits in the pancreas, gastric chief cells, Brunner's glands and, often, the liver, the siderosis is unlike that of human hemochromatosis[29,30,33,36] or thalassemia.[37] A pathogenesis related to that of the siderosis in the Bantu is unlikely. The distribution of the deposit is different,[34,38] and the mice received a nutritionally adequate diet of Purina Laboratory Chow in pellet form, containing 0.03 per cent iron, 0.9 per cent phosphorus, and 25 per cent protein. Idiopathic human hemochromatosis, siderosis in the Bantu, and

the siderosis in year-old mice share one feature in common not encountered in hemolytic disease.[29,30,32,34] All these have a conspicuous accumulation of lipofuscins in epithelium and macrophages. This similarity prompts the speculation that in these conditions, denatured (mitochondrial) hemin enzymes and lipoproteins aggregate to form the stainable cytoplasmic particles. Such a pathogenetic concept of siderosis in mice is in agreement with the views expressed by Sheldon[36] and Gillman[39] in relation to the human disorders.

Without dwelling on divergent views as to mast cell function or considering in detail the biochemical characteristics manifested by these cells,[40–44] their intimate association with iron and lipofuscin-laden macrophages in many organs of aged mice deserves attention. The distribution of the two cell types is so similar that they are often in contiguity. However, in such sites as the thymus, adrenal, ovary, testis and spleen, mast cells rarely accompany the abundant accumulations of lipofuscin or iron. Conversely many mast cells appear in the skin and gastric wall and somewhat fewer in peripheral nerves of young mice but are here unaccompanied by iron or lipid-containing macrophages.

In the cervical lymph nodes and uterus where these types of cells are most abundant, a large proportion of the mast cells stain with Alcian blue rather than safranin. Since considerable histochemical evidence suggests that in the Alcian blue-safranin procedure, safranin selectively stains mast cells containing mucopolysaccharides with abundant strongly acidic sulfate esters,[1] these findings imply depletion of sulfated mucins such as heparin in the areas densely infiltrated with macrophages. It is possible that mast cells phagocytize iron or lipid or that, alternatively, heparin functions in some manner to influence phagocytic processes. Heparin is known to form a complex with lipoproteins in the mast cell stroma and conceivably may do so with other extraneous substances. The characteristic capacity of iron salts to combine with acid mucopolysaccharide is the property utilized in the Hale stain for localizing acid mucins histologically. There is no indication, however, of metachromatic staining in the iron or lipofuscin-containing phagocytes, although after brief saponification lipofuscins in the lymph nodes and spleen exhibit orthochromatic azurophilia at pH 1.5.

Summary

Interstitial phagocytes containing stainable iron or insoluble lipid (lipofuscin) increase in number beneath most epithelial surfaces in the mouse as the animal ages. In the submaxillary gland, siderophages become more numerous in male than female mice at a year of age, whereas in the kidney the reverse is the case. Epithelium in the parotid, seminal

vesicle and mammary glands of older mice reveal both iron-containing cytoplasmic particles and bodies stained by the peracetic acid-aldehyde fuchsin (PAAF) procedure. PAAF-stained, iron-negative cytoplasmic particles, possibly composed of lipofuscin, are evident in the epithelium of the epididymis, vas deferens, prostate, coagulating gland, fallopian tube, liver and central neurons of year-old mice. In many tissues, the numbers of mast cells increase concurrently with siderophages and lipophages. The histochemical characteristics of the mast cells accompanied by abundant siderophages and lipophages indicate depletion of their sulfated mucopolysaccharides.

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LEGENDS FOR FIGURES

Figures 1a to 1f in color and 2 to 19 in black and white illustrate features observed in 10 to 18-month-old mice.

FIG. 1a. Cervical lymph node. Some mast cells are stained with Alcian blue and others by safranin. Alcian blue-safranin stain. × 210.

FIG. 1b. Inner muscle layer of the myometrium. Some Alcian blue and other safranin-positive mast cells are accompanied by occasional pigment-laden macrophages. Alcian blue-safranin stain. × 335.

FIG. 1c. Cervical lymph node. Iron-laden cells in the sinuses surround small pink-stained mast cells. The larger red-staining (strongly metachromatic) mast cells in the cords represent the type that fails to stain with Alcian blue. Ferrocyanide-safranin stain. × 385.

FIG. 1d. Myometrium. There is intimate intermingling of siderophages and mast cells. Ferrocyanide-safranin stain. × 530.

FIG. 1e. Tongue. Siderophages and mast cells appear in the connective tissue septum. Ferrocyanide-safranin stain. × 355.

FIG. 1f. Mammary gland. Iron deposits are manifest in and between epithelial and associated mast cells. Ferrocyanide-safranin stain. × 205.

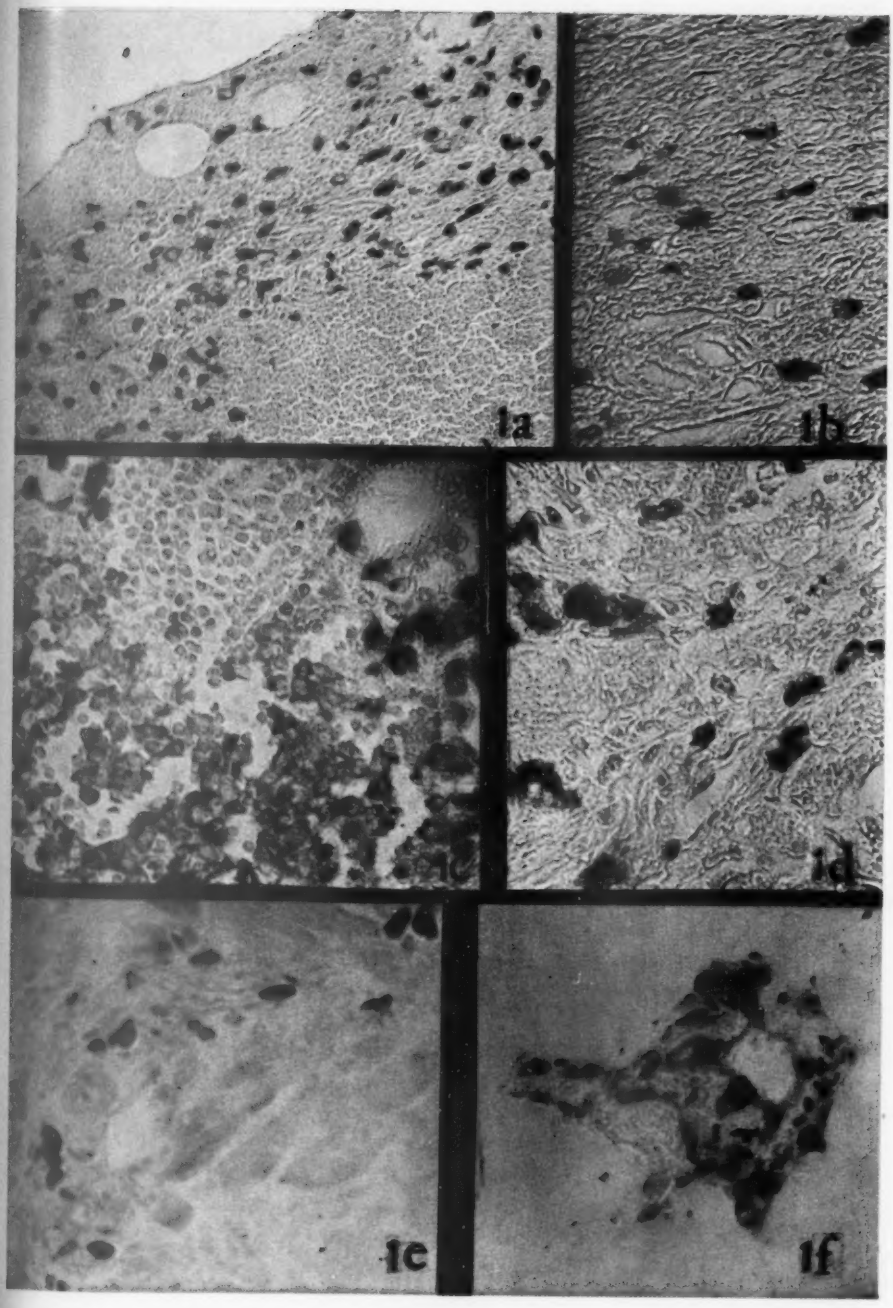
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FIG. 2. Siderophages in the submaxillary gland. Prussian blue stain. × 205.

FIG. 3. Stainable iron in the intercalated ducts and macrophages in the parotid gland. Prussian blue stain. × 385.

FIG. 4. Mast cells surround vessels and ducts in the hilus of the salivary gland. Azure A stain, pH 0.5. × 100.

FIG. 5. Siderophages in a section adjacent to that shown in Figure 4. Prussian blue stain. × 145.

FIG. 6. Siderophages in the lamina propria of the colon. Prussian blue stain. × 205.

FIG. 7. Heavy mast cell infiltration in the myometrium of an ex-breeder mouse. Cells near endometrium in the lower left-hand corner tend to stain less darkly. Azure A stain, pH 0.5. × 55.

FIG. 8. Iron-laden phagocytes in the endometrium and myometrium in a section adjacent to that shown in Figure 7. Prussian blue stain. × 63.

FIG. 9. Insoluble lipid in endometrial and myometrial macrophages. The section is adjacent to that shown in Figure 8. Sudan black stain. × 63.

FIG. 10. Stainable iron in interstitial macrophages of the epididymis. Ferrocyanide stain. × 205.

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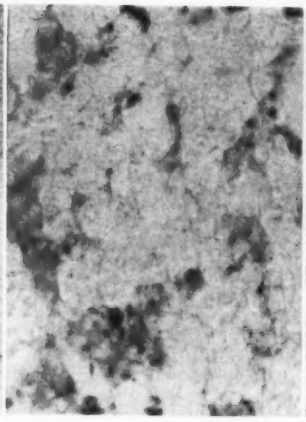

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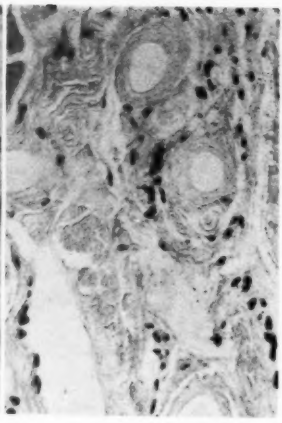

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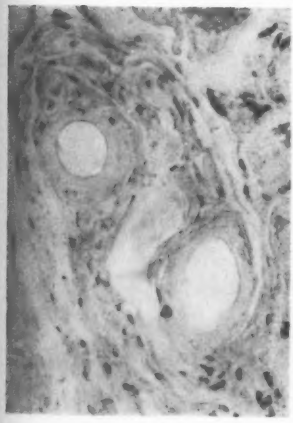

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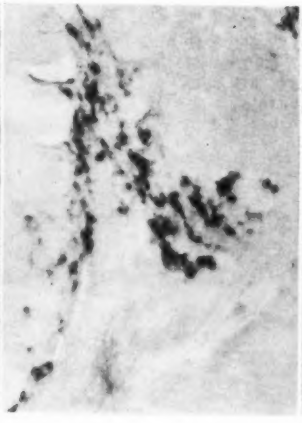

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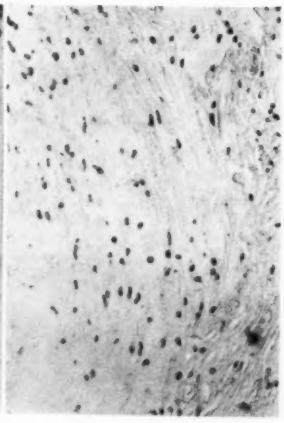

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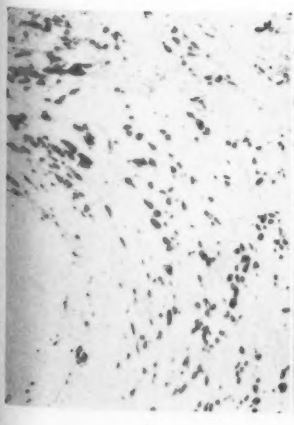

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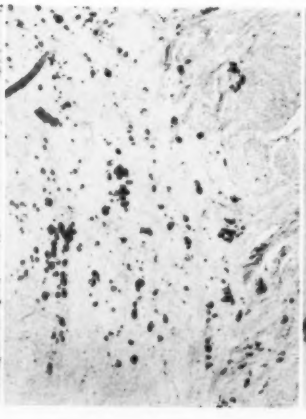

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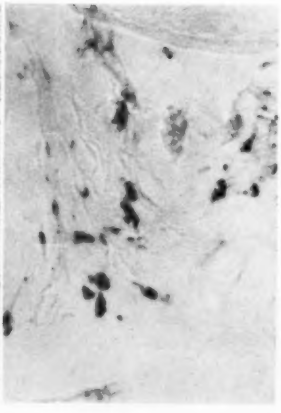

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FIG. 11. Intraepithelial cytoplasmic particles in the epididymis. Peracetic acid-aldehyde fuchsin (PAAF) stain. × 680.

FIG. 12. Mast cells in the wall of the ductus deferens. Azure A stain, pH 0.5. × 100.

FIG. 13. Siderophages in the wall of the ductus deferens in a section adjacent to that shown in Figure 12. Prussian blue stain. × 167.

FIG. 14. Cytoplasmic bodies in the epithelium of the vas deferens. This section is adjacent to that shown in Figure 13. PAAF stain. × 265.

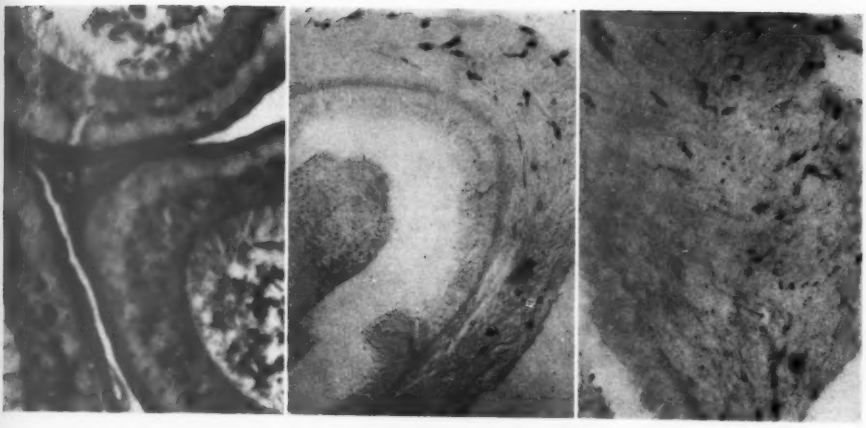
FIG. 15. Cytosiderin in the epithelium of the seminal vesicle. Prussian blue stain. × 465.

FIG. 16. Intra-epithelial cytoplasmic particles in a seminal vesicle. PAAF stain. × 680.

FIG. 17. Intra-epithelial cytoplasmic particles in the prostate. PAAF stain. × 680.

FIG. 18. Siderophages in the preputial gland. Prussian blue stain. × 110.

FIG. 19. Insoluble lipid in and between epithelial cells of a mammary duct. Sudan black stain. × 205.



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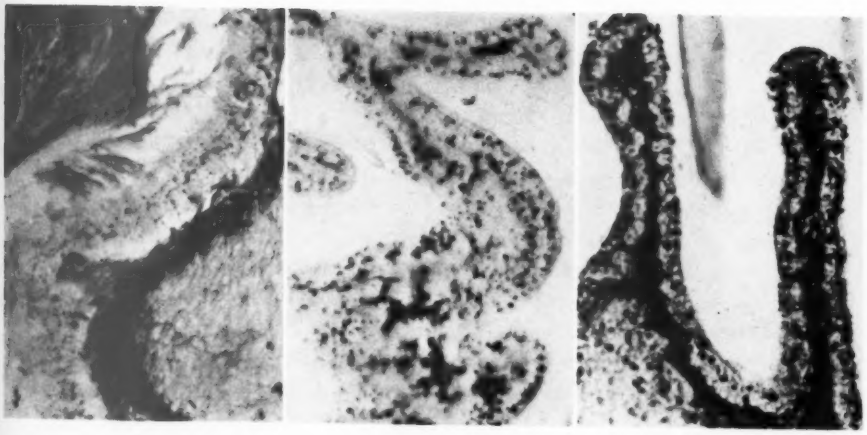

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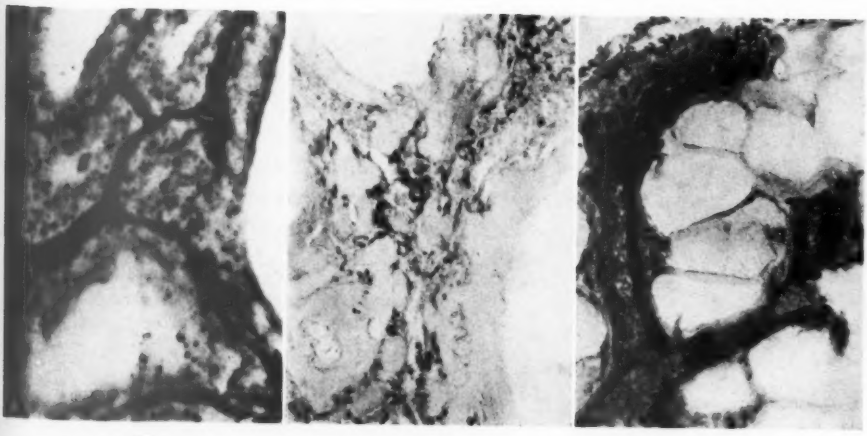

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EXPERIMENTAL INFECTION OF EMBRYONATED EGGS WITH CORYNEBACTERIUM DIPHTHERIAE

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The early students of diphtheria seem to have had the most success with the experimental production of the pseudomembranous lesion which is so characteristic of this disease. As noted in the monograph by Andrewes and his collaborators,[1] Loeffler achieved this in rabbits and other animals with the bacillus which he was the first to isolate in pure culture. Subsequent attempts to repeat these experiments have not succeeded so well. Most of the effort expended on diphtheria has been devoted to the development of knowledge regarding toxin and antitoxin, with its theoretical implications, and to its practical application in the treatment and prevention of the disease. Many unsolved problems remained for lack of a suitable experimental host in which the successive stages in the development of the pseudomembrane might be studied.

That the developing chick embryo would prove useful for this purpose became evident from Goodpasture and Anderson's[2] investigations of bacterial invasion in the chorio-allantoic membrane. Following them, Cromartie[3] succeeded in producing focal ulcerations in the respiratory and alimentary tract mucosa by inoculation of the amniotic cavity of 15- and 16-day embryos with a recently isolated strain of *Corynebacterium diphtheriae*. This study was also concerned with the factors which appeared to effect prevention of the lesions when embryos were passively protected with diphtheria antitoxin.

Others have directed their efforts toward adapting the chick embryo method for demonstrating the lethal effect of diphtheria toxin and the protective action of its antitoxin. Ozawa[4] investigated the effect of passively transferred antitoxin by way of the eggs of hens actively immunized with toxoid. Evans[5] demonstrated the susceptibility of embryos to the lethal effect of toxin and showed that the method was useful for identifying toxigenic strains of Corynebacteria.[6,7] More recently Dishon[8] has advocated the use of embryonated eggs for standardizing antitoxin.

The experience with an outbreak of diphtheria a few years ago revived

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the interest of one of us (G.J.B.) first aroused by close association with the early work on *C. diphtheriae* in chick embryos. A renewed effort was made to obtain a more accurate estimate of the lethal effect of diphtheria toxin on eggs at various stages of incubation. The acquisition of a number of strains of *C. diphtheriae* isolated from cases during the outbreak led to experimentation with their behavior in embryonated eggs. A method was discovered whereby pseudomembranous lesions could be produced on the chorio-allantoic membrane. This made possible a detailed bacteriologic and histologic study of successive phases in the pathogenesis of the local lesion and its effect on the developing embryo. The observations made during these experiments constitute the subject matter of this report.

Material and Methods

Embryonated Eggs

White Leghorn eggs from a single poultry farm maintaining flocks under uniform feeding and housing conditions were kept in a regular hatching incubator for the appropriate time and prepared by the "window" method 24 to 48 hours prior to inoculation. All inoculations were performed by dropping measured amounts of diphtheria toxin or cultures of *C. diphtheriae* on the surface of the exposed chorio-allantoic (C-A) membrane. Since differences in size were found to affect susceptibility, eggs from 50 to 55 gm. in weight only were used for the toxin LD_{50} titrations. Seasonal variations in the incidence of spontaneous deaths were avoided as much as possible by performing the definitive determinations for susceptibility to toxin during a 4-week period from May 15 to June 15. Experiments on infection with strains of *C. diphtheriae* were performed at various times during the year.

Diphtheria Toxin

A stable standardized diphtheria toxin (obtained through the kindness of Dr. C. G. Culbertson of the Lilly Research Laboratories, Indianapolis, Indiana) containing 30 M.L.D. for guinea pigs per ml. was used in all of the LD_{50} titrations.

Corynebacterium Diphtheriae Strains. The American Type Culture Collection strains *C. diphtheriae,* type *gravis*, no. 9674; type *intermedius*, no. 9675; type *mitis*, no. 9673 and *Park 8*, no. 296, were compared for their effect on embryonated eggs with several strains isolated from cases of diphtheria occurring during the outbreak in 1954. From the latter group, one strain was arbitrarily chosen and used in the experiments in which the development of pseudomembranes on the C-A membrane was studied. This strain had been submitted for identification to Dr. Elizabeth Parsons of the Communicable Disease Center. She designated it as *C. diphtheriae, gravis-like*. A large number of vials of lyophilized samples prepared from a single 24-hour culture in brain-heart infusion with 10 per cent serum served as a uniform source of inoculum for the various experiments.

Procedures for the Study of C-A Membrane Infection

The medium in which the inoculum was cultured consisted of brain-heart infusion broth (Difco) to which 10 per cent by volume of normal inactivated rabbit serum was added. A loopful of actively growing culture was transferred to 150 by 16 mm. tubes containing 10 ml. of the medium and then incubated at 37° C. for 36 to 48 hours. The cultures were then washed by centrifugation and resuspension in 15 ml. amounts

of fresh brain-heart infusion broth 5 successive times. They were then reconstituted to their original 10 ml. volume.

Because of the clumping character of *C. diphtheriae*, accurate estimates of the number of viable bacilli in the standard 0.1 ml. inoculum could not be made. By means of pour plates, rough average colony counts of 10^6 to 10^8 per 0.1 ml. were obtained. With this relatively large number of washed micro-organisms, it was found that pseudomembrane-like lesions would develop on most of the C-A membranes when inoculated on the 14th day of incubation. The C-A membrane of younger embryos very seldom developed grossly visible inflammatory reactions to infection with *C. diphtheriae*.

After inoculation, the course of the infection was observed in the following manner. Groups of 3 to 5 living embryos were chosen arbitrarily at 3, 6, 12, 24, 48, 72, 96 and 120 hour intervals for bacteriologic and histologic investigation. At each of these periods, samples were removed with as little injury as possible from the surface of the C-A membrane, by means of a sterile inoculating loop, for culture and for preparation of methylene blue stained smears. The exposed membrane was fixed in Zenker's (with 5 per cent acetic acid) solution. The accompanying embryo was immersed in Zenker's solution for 2 minutes, after which the heart was exposed. By means of a Pasteur capillary pipette, a small sample of blood was then withdrawn for bacteriologic culture. After proper fixation, the membranes and embryos were processed for sectioning and staining for histologic examination. Sections were stained with hematoxylin and eosin, and for satisfactory differentiation of bacteria and the cells of the inflammatory reaction, selected sections were stained with Wright's stain by the method described by Goodpasture.[9]

An inoculating loopful of exudate or a light scraping was removed from the surface of the C-A membrane for culture on blood agar from each of the embryonated eggs remaining at 24, 48, 72, 96, and 120 hours. At each of these intervals the dead embryos were removed from the egg and a sample of heart blood obtained for culture to determine whether invasion of the blood from the C-A membranal surface had taken place. Each of the strains of Corynebacteria obtained by culture from the C-A membranes of embryos sacrificed for study, from the C-A membranes of those that died and that survived to the fifth day after inoculation were tested for toxin production by intracutaneous injection in rabbits. This test was performed with 0.1 ml. amounts of 48-hour brain-heart infusion broth cultures injected 4 hours before administering sufficient antitoxin intravenously. The repeat injection with the same amount from each culture was made 3 hours later to demonstrate the specificity of the resulting reactions.

Observations and Results

The LD_{50} of Diphtheria Toxin for Embryonated Eggs at Various Stages of Incubation

Embryonated eggs were inoculated by dropping graded doses of diphtheria toxin on the C-A membrane during the ninth, eleventh, 13th and 15th days of incubation and returned to the incubator at 37° C. The results of these experiments are presented in Table I. The method of Reed and Muench[10] was followed in the calculation of the LD_{50} values and are expressed in fractions of the previously determined M.L.D. for guinea pigs.

A marked gradient in decreased susceptibility to the lethal effect of toxin with increase in age of embryonated eggs is evident from these

TABLE I

THE LETHAL EFFECT OF GRADED DOSES OF DIPHTHERIA TOXIN ON THE CHORIO-ALLANTOIS ON EMBRYONATED EGGS AT VARIOUS STAGES OF INCUBATION

No. of days incubation	Days after inoc.	Diphtheria toxin in guinea pig M.L.D.														Norm. contr.*	LD_{50} in guinea pig M.L.D.
		1	3/4	1/2	1/3	1/5	1/10	1/20	1/30	1/40	1/60	1/80	1/120	1/160	1/240		
9	1				3/5†	0/5	1/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	
	2				5/5	5/5	5/5	4/5	4/5	3/5	0/5	1/5	0/5	0/5	0/5	0/5	
	3							5/5	4/5	3/5	4/5	4/5	0/5	0/5	0/5	0/5	1/115
	4								4/5	4/5	5/5	5/5	1/5	1/5	0/5	0/5	
Survivals					0	0	0	0	1	1	0	0	4	4	5	5	
11	1			0/3	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5		0/5	
	2			3/3	5/5	5/5	2/5	1/5	0/5	1/5	0/5	0/5	0/5	0/5		0/5	
	3						4/5	4/5	4/5	3/5	1/5	0/5	0/5	0/5		0/5	1/52
	4						5/5	4/5	5/5	3/5	2/5	2/5	0/5	0/5		0/5	
Survivals				0	0	0	0	1	0	2	3	3	5	5		5	
13	1			1/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5		0/5	
	2			5/5	5/5	4/5	3/5	0/5	1/5	1/5	1/5	0/5	0/5	0/5		0/5	
	3					4/5	3/5	0/5	1/5	1/5	0/5	0/5	0/5	0/5		0/5	1/15
	4					4/5	4/5	1/5	1/5	1/5	0/5	0/5	0/5	0/5		0/5	
Survivals						1	1	4	4	4	5	5	5	5		5	
15	1	1/5		0/5	0/5	0/5	0/5	0/5	0/5	N.D.‡	0/5	0/5				0/5	
	2	3/5		5/5	5/5	5/5	2/5	1/5	0/5		1/5	0/5				0/5	
	3						4/5	2/5	1/5		1/5	0/5				0/5	1/13
	4						4/5	3/5	1/5		1/5	0/5				0/5	
Survivals		2		0	0	0	1	2	4		4	5				5	

* Normal controls.

† Numerator: accumulative deaths
Denominator: number inoculated

‡ N.D.: not done

determinations. These differences cannot be explained on the basis of reduced permeability of the C-A membrane with increased age or by other factors which might interfere with diffusion or absorption from this site. Sufficient comparative experiments were done with subamniotic and other routes of injection to establish the fact that the increase in the LD_{50} reflects a real increase in resistance with age of the developing embryo to the lethal effect of the toxin.

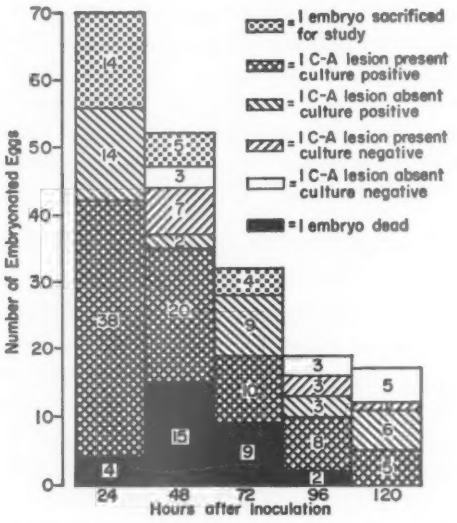
The uniformly high susceptibility of 9-day embryonated eggs, noted particularly by Evans,[5] is especially apparent. Since the LD_{50} for 15-day embryos appears to be approximately 10 times greater than for 9-day embryos it is evident that the stage of incubation must be taken into account if this method is to be used for toxin titrations or antitoxin standardization. The foregoing determinations prove to be of considerable interest in their bearing on the study of the pathogenesis of active infection of the C-A membrane with *C. diphtheriae*.

C. diphtheriae Infection of the C-A Membrane and Its Effect on Embryonated Eggs

Laboratory Strains. The American Type Culture Collection strains *C. diphtheriae* types *gravis, intermedius, mitis,* and the *Park 8* could not be adapted to growth on the surface of the C-A membrane of embryonated eggs of 9, 11, 13 and 15 days' incubation. Lethal effects were obtained only when unwashed cultures were introduced, under which circumstances the preformed toxin proved to be the active agent. With washed cultures no deaths occurred. With either type of inoculum, the type laboratory strains failed to multiply and establish infection. This was previously noted by Goodpasture and Anderson.[2]

Recently Isolated Strains. Each of 5 strains isolated from cases during the 1954 outbreak were found to multiply readily on the surface of the C-A membrane of 9, 11, 13, and 15-day embryonated eggs. All of the younger embryos died within 48 hours; among those of 13- and 15-day incubation a small number would survive 96 hours, but no longer. Infection of the membrane of the older embryos occasionally resulted in the formation of visible pseudomembrane-like lesions. This circumstance led to the use of thoroughly washed bacilli for the inoculum in order to avoid the immediate injurious effect of preformed toxin and provide the opportunity for the infection to proceed in direct consequence of the multiplication of the micro-organisms. The following account will present the progress of a single final experiment with infection of the C-A membrane of 14-day embryonated eggs, utilizing an inoculum prepared with washed *C. diphtheriae*. This was obtained from a 36-hour serum-broth culture

of the strain which, as previously indicated, was arbitrarily chosen from those isolated during the 1954 outbreak. The course of this experiment and its effect on 70 embryonated eggs is graphically represented in Text-figure 1. As the legends indicate, the number of embryonated eggs on which certain definite observations were made on each of the successive 5 days following inoculation are shown. If the 23 sacrificed for microscopic examination are not taken into account, it may be con-



TEXT-FIGURE 1. The effect of infection of the C-A membrane with *C. diphtheriae* on 14-day embryonated eggs.

sidered to illustrate the course of experimental diphtheria in 47 embryonated eggs. As such, however, it provides only a partial insight into the dynamics of this infectious process.

The 30 deaths which occurred can be ascribed solely to the effect of toxin elaborated during the progress of the infection on the surface of the C-A membrane. Blood stream invasion and localization within the embryo by *C. diphtheriae* did not take place. No bacteria could be cultured from the heart blood of each of the dead embryos or from the heart blood of each of those sacrificed for study. There were no focal or generalized inflammatory reactions in any of the embryos sacrificed in this or in many of those from other experiments. There were only two embryos in which small foci of myocardial necrosis were present. Otherwise, localized or widespread hemorrhage, indicative of vascular

injury of the type produced when diphtheria toxin alone is administered, was the only lesion encountered.

The survival at 5 days following inoculation of 17 of the 47 (30 per cent) is perhaps the most startling feature of this experiment. The foregoing LD_{50} determinations indicate that a relatively small amount of toxin is required to kill 15- to 16-day embryonated eggs. Either less than the lethal dose was produced or, if more, it either did not diffuse through the membranal lesion or the embryos which survived were insusceptible to diphtheria toxin. It is highly unlikely that antitoxin formation takes place at this stage of embryonic development. Cultures of Corynebacteria obtained from the C-A membrane of each of 11 of the survivals were found to contain toxin by intracutaneous tests in rabbits to the same degree as those obtained from the membranes of each of the 30 embryos that died. Corynebacteria could not be obtained by culture from the C-A membrane of the 6 others that survived.

An elucidation of the various factors which participate in the dynamics of this infectious process requires a somewhat detailed account of the observations that were made. A correlation of what was found by culture, methylene blue smears and microscopic section at successive intervals provides a partial explanation for the complete disappearance of Corynebacteria from 6 and the survival of both the bacteria and the embryo in 11 eggs. These host-parasite inter-reactions appear to have proceeded in the absence of what is usually considered as an immune response.

The first day: During the first day 4 embryos died. Grossly visible reactions other than capillary dilatation did not develop in any of the C-A membranes during the first 12 hours. Exudative and pseudomembrane-like lesions became visible in 46, including the 4 that died during the 12 to 24 hour period (Figs. 1 and 2). No reactions were present in 14 of the 60 remaining at 24 hours. During this time the C-A membranes only from 10 living embryos were fixed for study; 3 at 3 hours, 3 at 6 hours, and 4 at 12 hours. Cultures from these 10 membranes and from 60 remaining at the end of 24 hours were positive for Corynebacteria, indicating that infection had been established in each of the 70 embryonated eggs inoculated at the beginning of the experiment. At 24 hours the C-A membranes and accompanying embryos of 4 with gross lesions were placed in fixative.

At the 3-hour interval, methylene blue-stained smears from each of the 3 selected for examination revealed characteristic bacilli in typical clumps and in sufficient numbers to indicate that rapid multiplication was in progress (Fig. 4). At this time very few inflammatory cells were encountered in the smears. In sections of the C-A membranes there was no evidence of injury to the ectodermal epithelium (Fig. 3). Marked swelling due to edema and vascular dilation characterized the mesodermal layer. Numerous polymorphonuclear cells showing no signs of injury were scattered throughout the intercellular spaces and were beginning to concentrate in considerable numbers beneath and in small numbers on the surface of the ectodermal epithelium. There was no evident disturbance of the endoderm.

Methylene blue smears prepared from the surface of each of the 3 C-A membranes

selected after 6 hours had elapsed showed a great reduction in the number of bacilli as compared with the 3-hour period. Most of the micro-organisms were phagocytized by about 1 in 20 of the abundant normal-appearing neutrophils which comprised the preparations (Fig. 6). The bacilli were for the most part swollen, poorly stained or fragmented. Occasional extracellular clumps of short, deeply stained organisms appeared to be actively multiplying.

The sections through each of the membranes fixed at 6 hours showed several extensive areas in which the ectodermal epithelium had been destroyed. The denuded surface was covered with a heavy layer of exudate composed of well preserved neutrophils (Fig. 5). Careful search disclosed an occasional cell containing phagocytized bacilli. The mesodermal layer was markedly edematous and the blood vessels were considerably dilated. Occasional clumps of red cells appeared adjacent to veins. Relatively few inflammatory cells were encountered in the intercellular spaces. The endodermal layer was normal.

Smears prepared from each of the 4 C-A membranes just before fixation at the 12-hour interval showed that the inflammatory cells had undergone considerable degeneration. Most of them appeared as amorphous smudges; the remainder were clumped together with indefinite outlines and poorly stained structural detail. No phagocytosis was observed, but large numbers of short, deeply staining coccobacillary forms were present and appeared to be actively multiplying in the cellular debris. Occasional normal-appearing neutrophils which had not phagocytized bacilli were encountered.

Sections of these membranes showed the ectodermal layer to be intact. There was evidence that regenerated epithelium had covered previously ulcerated areas. This repair appeared to have taken place beneath the layers of exudate that covered the membranal surface. Immediately adjacent to the outer ectoderm the exudate was made up of a thin layer of acidophilic, amorphous or hyalinized cellular debris. To the outside of this a layer of varying thickness was composed of coagulated clumps of inflammatory cells mixed with erythrocytes. Interspersed among the degenerating exudate were numerous well preserved polymorphonuclears. In the outermost zone of the exudate well stained masses of micro-organisms were irregularly scattered. The intercellular spaces in the mesoderm were expanded widely and contained many neutrophils which appeared to be new arrivals. The veins in particular were much dilated, but there were no signs of hemorrhage. The endoderm appeared to be unaffected.

Examination of the methylene blue-stained smears prepared from the membrane of each of the 4 embryonated eggs sacrificed at the 24-hour interval revealed an increase and intensification of the reaction in progress at 12 hours. There was a diffuse background of smudges and cellular debris. Many faintly outlined cells were stuck together in clumps and masses. Within some of these masses were groups of deeply stained bacilli which apparently had been multiplying rapidly (Fig. 8). In addition there were numerous normal-appearing polymorphonuclears, some of which had phagocytized several bacilli.

In the sections (Fig. 7) from 2 of the 4 sacrificed, the ectodermal epithelium was intact. It was overlaid by a thick layer of exudate composed of what appeared to be several strata. The layer immediately adjacent to the epithelium was quite thin but densely hyaline. Overlying this were 2 or 3 additional distinct layers, each less coagulated, amorphous and hyaline than that directly beneath it. The outer zone was composed of loosely aggregated masses of amorphous cellular debris and indistinctly outlined inflammatory cells. Among these were numerous viable polymorphonuclears. At the outer edge of the coagulated exudate many clumped masses of intensely stained bacteria were scattered. The mesoderm was quite edematous and many well preserved neutrophils appeared in intercellular spaces. There was no evidence of vascular injury in the form of hemorrhage or thrombosis.

The membranes of the other two embryos sacrificed at this time showed the same type of exudate on the surface, but there were wide areas of ectodermal epithelial destruction.

The mesodermal veins especially showed evidences of injury in the form of widespread hemorrhage and the accumulation of thrombocytes on their endothelial surfaces. The embryos accompanying these membranes contained numerous small hemorrhages scattered widely throughout striated muscles and subcutaneous tissues. No myocardial lesions were apparent. Sections of the two embryos accompanying the membranes without ectodermal ulceration showed no hemorrhage.

The second day: Starting with 52 living embryonated eggs, the second day following inoculation was marked by the greatest number of deaths. Of the 15 deaths 11 had already developed exudative or pseudomembrane-like reactions before the end of 24 hours. The remaining 4 died without any gross evidence of reaction in the C-A membrane. Of the 37 alive at the end of 48 hours, there were 32 with gross lesions at the end of the first day. Thus, by the end of the second day all but 4 of the 56 embryos alive at 24 hours had developed visible reactions in the C-A membrane. The 4 that showed no reactions died within 48 hours. The gross lesions at the end of the first day had disappeared from 5 at the end of the second day. From 3 of these no Corynebacteria could be cultured; from the other 2, the cultures were positive. Of the 32 with gross lesions at 48 hours, there were 25 from which Corynebacteria could be recovered by culture. None grew out from the remaining 7. Five C-A membranes and their accompanying embryos were prepared for study at the end of 48 hours. Corynebacteria could be cultured from 3 of these but not from the other 2.

Methylene blue stained smears of the C-A membrane exudate in 3 eggs from which Corynebacteria were cultured contained masses of inflammatory cells which stained poorly and had indistinct features. Scattered throughout the smears was a moderate number of clearly outlined polymorphonuclears, some of which contained poorly stained, fragmented, phagocytized bacilli. There was also a moderate number of clumps of well stained bacilli which appeared to have been multiplying actively.

The sections through the membranes of 2 of these 3 showed a considerable amount of ectodermal ulceration. Overlying this was a thick layer of exudate composed of several strata. Immediately adjacent to the ulcerated surface, the exudate was coagulated and hyalinized. Overlying the strata were irregularly scattered clumps of intensely stained bacteria. The outer layer was composed of masses of exudate containing cells in which some of the morphologic details were still visible. The mesoderm contained much active inflammation, and considerable injury to the lining of the dilated veins was manifested by hemorrhage and margination of thrombocytes. In the embryos accompanying these membranes, numerous scattered hemorrhages were noted in striated muscle and subcutaneous tissues.

The C-A membrane of the third embryonated egg revealed an exudative reaction of the same type as in the other 2 in this group. Overlying the layer of hyalinized exudate was an almost unbroken zone of deeply stained bacteria which in turn was covered by patches of more recently accumulated inflammatory cells. In contrast to the others, the ectodermal epithelium was everywhere intact. The mesoderm was edematous and contained a moderate number of leukocytes. Injury to the vascular bed was apparently absent or minimal. There were no hemorrhages or thrombosed vessels, nor had margination of thrombocytes taken place. The related embryo exhibited no hemorrhages or other evidences of injury.

The C-A membrane of the 2 embryos sacrificed at 48 hours, from which no Corynebacteria could be cultured, had very little exudate on the surface. For the most part it was dense and hyaline with only a few patches of recognizable cells. No bacteria were found. The ectodermal epithelium was intact along the entire extent of the sections. The mesoderm was only slightly distended by edema. The blood vessels

were not dilated, and there was no hemorrhage. Numerous active leukocytes were present in the extracellular spaces but they did not appear to be migrating to the membranal surface. The accompanying embryos had no lesions or reactions of acute inflammatory or hemorrhagic nature.

The third day: At the beginning of the third day there were 32 living embryonated eggs. During the course of this day, 9 died. Each of the dead had pseudomembrane-like reactions on the C-A membrane. Corynebacteria were recovered by culture from the membrane of all 32 eggs at the end of 72 hours. Of the remaining 23 living there were 9 from which most or all of the membrane reaction had disappeared. The 14 others had pseudomembrane-like lesions (Fig. 9). It is of interest to note that the 3 eggs from which the C-A membranal reaction had disappeared and no bacteria were recovered by culture at the end of the second day, each again had developed membranal exudate from which Corynebacteria could be cultured at the 72-hour interval. At this time 4 embryonated eggs with grossly visible reactions were prepared for microscopic study.

The methylene blue stained smears of the exudate on the C-A membrane of 3 of the 4 sacrificed had the same characteristics. There were many clumps of amorphous cellular debris and masses of poorly defined leukocytes that were stuck together. In addition there were large numbers of well preserved polymorphonuclears which had not phagocytized the numerous typical uniformly distributed Corynebacteria.

The sections of the membranes in each of these were quite striking in appearance (Fig. 10). Ectodermal epithelium had been destroyed for the most part. In its place there was a thick layer of exudate composed of leukocytes and numerous red cells in various stages of disintegration. In the exudate there were many clearly recognizable polymorphonuclears. In the middle zone of this exudate there was a distinct layer composed of masses of deeply stained bacteria. The mesoderm was greatly distended by edema, inflammatory infiltration and vascular dilatation. Dilated and engorged veins especially showed much evidence of injury. Some were the seat of thrombosis; in others, marginating thrombocytes sharply outlined the endothelial surface. There was considerable fibroblastic proliferation in scattered foci. All types of cells appeared to be seriously injured as evidenced by acidophilic staining. The accompanying embryos showed numerous hemorrhages in striated muscle and subcutaneous tissue. There were small focal areas of necrosis in the myocardium of 2. Loss of striation, evident coagulation of muscle fibers at these points, and infiltration by small numbers of neutrophils were readily appreciated.

The methylene blue smear from the C-A membrane of the remaining embryo sacrificed at 72 hours was in marked contrast to those from the others. Most of the exudate consisted of clumps of amorphous or degenerating inflammatory cells, among which were many clumps of swollen vacuolated leukocytes. Some of them had phagocytized considerable numbers of short, poorly stained bacilli. None of the bacteria were extracellular.

The membrane with this reaction showed its surface exudate to consist mostly of amorphous debris which had become coagulated and hyaline in the layers directly adjacent to the surface. This was everywhere intact. No bacteria were encountered. The mesoderm was moderately swollen, and only a few neutrophils were seen in the intercellular spaces. There were a few foci exhibiting an increase in fibroblastic proliferation. The blood vessels were not dilated and showed no evidence of injury. Sections through the accompanying embryo were free of lesions.

The fourth day: Two embryos died during this day, leaving 17 alive. The C-A membrane of the 2 that died had pseudomembrane-like reactions and Corynebacteria were recovered by culture. Grossly visible inflammatory reactions were present in the C-A membranes of 11 of the 17 survivors. From 8 of these, Corynebacteria were recovered; the other 3 yielded negative cultures. Of the 6 remaining, the gross exudate

had disappeared from the C-A membrane. From 3 of these, Corynebacteria were recovered. Cultures from the other 3 were sterile.

Methylene blue-stained smears prepared at this stage contained mostly cellular debris and no recognizable bacilli. Sections of the C-A membrane of surviving embryos collected during previous experiments showed nothing but varying thicknesses of stratified hyalinized exudate over the surface. The ectoderm was usually intact, the outer layers showing considerable keratinization and varying numbers of "epithelial pearls" were encountered. There was very little if any inflammatory reaction in the mesoderm. Most of the sections showed considerable fibroblastic proliferation in areas which appeared to represent previous sites of inflammation. No signs of vascular injury were evident at this stage. The accompanying embryos were in no way remarkable.

The fifth day: The 17 embryos surviving the fourth lived through the fifth day. Membranal lesions were present in 6, of which 5 yielded positive cultures and 1 was negative for Corynebacteria. The gross inflammatory reaction had disappeared from the 11 others. From 6 of these the cultures were positive. The findings in the smears were essentially the same as those described on the fourth day. Sections from membranes and embryos sacrificed in other experiments showed no remarkable differences in microscopic characteristics from those described for the fourth day .

Discussion

Many of the features characteristic of diphtheria are experimentally reproducible in embryonated eggs. The pseudomembrane-like reaction of the C-A membrane is in many respects similar to the local oropharyngeal or cutaneous lesion. The toxin produced by *C. diphtheriae* growing near the surface exerts its injurious effect locally and by way of the circulation at various sites in the developing embryo in much the same way as the disease progresses in the human host.

As was observed many years ago,[2] attempts to induce stock laboratory strains to grow in embryonated eggs proved unsuccessful. They also failed in the present studies. The experimental infection could be established only with strains that had been isolated relatively recently from cases during an outbreak of diphtheria. In order to promote the development of the pseudomembrane-like lesion on the chorio-allantois, it was found necessary to remove as much as possible of the preformed toxin from the cultures that were used as the inoculum. When toxin is present in the inoculum, its immediate injurious effects at the site of inoculation presumably provide the infecting micro-organism with conditions favorable to rapid multiplication. In consequence, toxin production is accelerated, the inflammatory response to the presence of the bacilli is inhibited, and the mortality rate will practically always be 100 per cent within 48 to 72 hours.

In response to the inoculation of the surface of the chorio-allantois with a relatively large dose of bacilli washed free of toxin, an acute inflammatory response develops coincidental with the early stages of bacterial growth. Within 3 to 6 hours, rapid bacterial multiplication takes

place, and large numbers of polymorphonuclears accumulate at the site of inoculation. In the beginning, all the bacteria are extracellular, but toward the end of 6 to 8 hours the majority of them have been phagocytized. Apparently most of the phagocytized bacilli are destroyed. At this stage in the reaction, degeneration and necrosis of the inflammatory cells that have accumulated on the membranal surface sets in. Whether this is brought about by toxin liberated by phagocytized bacilli or by toxin elaborated before phagocytosis, or by the relatively few remaining extracellular micro-organisms cannot be determined from the observations that were made. For a short time, at least, the necrotic cellular debris seems to serve as an excellent growth medium so that a new wave of rapid bacterial multiplication ensues. This is turn brings a marked increase of new phagocytes into the area. In the meantime the first accumulation of inflammatory cells undergoes coagulation and hyalinization. Thus, these reciprocating phases of rapid bacterial multiplication and inflammatory reaction succeed one another at 6- to 12-hour intervals. In this manner the pseudomembrane-like lesion becomes stratified, with the bacteria maintaining their position near the surface of the reaction. It is this process which appears to underlie the eventual survival of a certain proportion of the embryonated eggs. In some of them the succession of events seems to promote conditions which are unfavorable to bacterial multiplication so that phagocytic activity becomes preponderant and all viable bacteria disappear. In others either the development of the inflammatory lesion seems to slow down the bacterial growth rate, thus depressing or inhibiting toxin production, or a membrane develops, through which toxin cannot diffuse. As Dubos[11] has emphasized, the rapidly developing biochemical and physical alterations in inflammatory exudates promote an environment that is often deleterious to the activity and survival of many kinds of microbes. These aspects of the problem would seem to be readily accessible to further investigation.

It is diphtheria toxin that killed the embryonated eggs in this experiment. When toxin diffuses from the bacilli on the surface through the exudate and into the chorio-allantoic mesoderm, the vascular bed is injured, with resulting thrombosis and hemorrhage. When the toxin in the embryonic circulation reaches a level within the range of that produced by the administration of an average M.L.D., the embryo dies with widespread hemorrhage. Evidences of injury to myocardial fibers are occasionally encountered. There also may be injury to the nervous system, but this is not apparent in the microscopic sections.

The amount of toxin required to kill 14- to 16-day-old embryos is relatively small, but it appears more likely that the rate of its production by the bacterial population is more important in the cause of death. When produced at a rapid rate, toxin depresses the inflammatory re-

sponse, and the leukocytes that wander into the area are quickly destroyed. This appears to favor bacterial proliferation and accelerated toxin production. These considerations have been stressed by Cromartie [8] in relation to his studies with diphtheria induced in chick embryos by subamniotic inoculation.

The strain of *C. diphtheriae* used in these experiments appears to produce toxin at a relatively slow rate. This circumstance presumably promotes a succession of reciprocal phases of bacterial growth and inflammatory response in which the balance in the majority of instances results in the predominance of bacterial proliferation and toxin production. In the surviving embryonated eggs, on the other hand, the inflammatory reaction becomes predominant, and the changes it undergoes seem to be inimical to bacterial proliferation or toxin production. There is no evidence to conclude that survival is effected by a selective process imposed by the inflammatory reaction whereby all toxin-producing elements of the bacterial population are eliminated. The toxigenicity tests on cultures obtained from the surviving embryos proved them to be equal in toxin production to cultures derived from embryos dead from the infection.

The progress of this experimental infection, with its constantly shifting balance between preponderance of bacterial multiplication on the one hand and the intensity of the inflammatory response on the other, provides certain insights into the phenomenon of host and parasite population inter-reaction. In many respects 14- to 16-day-old embryonated eggs may be considered to make up a fairly uniform host populace. This is perhaps most pronounced in the sense that immunity in the form of specific antitoxin production does not play a part in the experimental infection with *C. diphtheriae*. Nor does it seem reasonable to assume that whatever constitutes species or innate immunity is involved in the process which results in the survival of a considerable proportion of a group of 50 or more embryonated eggs inoculated on the chorio-allantois. Furthermore, there is very little variation in the size of the lethal dose of toxin required for eggs of approximately the same weight.

Thus it is evident that *C. diphtheriae* washed free of toxin will proliferate on the surface of the chorio-allantois of 14-day embryos. In response to whatever irritant or stimulus is exerted by the growing bacterial population, an inflammatory exudate collects at this focus. If there were exact uniformity in every respect in the conditions of the chorio-allantoic surface from one egg to the next and also an exact uniformity in composition of the number, growth rate potential and toxin-producing capacity of the bacterial population in the inoculating dose, the eventual outcome of the infection would more likely be the same in each instance.

It would be most unusual to succeed in the preparation of a large

number of embryonated eggs in such a way that the site to be inoculated would present uniform conditions for bacterial proliferation. Variations in the number of necrotic cells, the effects of hemorrhages and thrombosis and other small differences in the extent of the injuries from egg to egg during the process of exposure of the C-A membrane are unavoidable. As a result, each egg presents the infecting dose of bacteria with slightly different initial conditions. These factors in all likelihood profoundly affect the rate of bacterial growth in the early stages of infection, determining in the case of *C. diphtheriae* the efficiency and rate of toxin production.

Compared with well established laboratory strains which presumably have a homogeneous composition, strains recently isolated from the natural disease are more likely to be extremely heterogeneous. The marked variation in toxin-producing capacity of different strains of *C. diphtheriae* has long been recognized. The more recent demonstration that toxin-producing capacity of individual organisms is associated with prophage B lysogenicity,[12] introduces another important variable. Recently isolated "wild" strains are perhaps not composed entirely of lysogenic individuals. Their proportion may vary from strain to strain as well as from one infecting dose to the next. These variations would contribute to the different progress of infection noted in the different individuals of a large group of embryonated eggs. In this regard, further experiments with pure line clones of lysogenic and nonlysogenic bacilli would prove to be of much interest.

This experimental approach is also an exercise in epidemiology. It reduces an epidemic to some of its simpler and more basic components. It is rather presumptuous to present the embryonated egg population in terms of resistance or susceptibility. Neither is it especially enlightening in this connection to consider the *C. diphtheriae* population in terms of invasiveness or predatory propensities. Under the conditions described *C. diphtheriae* populations will multiply when placed on the surface of the chorio-allantois of 14-day embryonated eggs. The growing parasite population stimulates an inflammatory reaction in the embryonic host. There is initiated a series of fluctuating conditions conducive to rapid multiplication and accelerated toxin production on the one extreme and inhibition or depression of growth or of toxin production on the other. The eventual outcome is presumably determined by the composition of the parasite population in the inoculum and of the physical and physiologic conditions of the chorio-allantoic surface at the site and time of inoculation. Much of this could be measured, analyzed, and more carefully evaluated. The outcome of the experimental disease in the individual or in the group could then be expressed in an equation in which the

various reaction factors would find their proper position. In this manner the terms "resistance," "susceptibility," "defense," "invasiveness," "virulence," "tolerance," and many other concepts pertaining to the infectious process would acquire a more precise meaning.

Summary

The LD_{50} in fractions of a guinea pig M.L.D. was determined for embryonated eggs of 9, 11, 13, and 15 days' incubation. A gradient of reduced susceptibility to the lethal effect of toxin in direct proportion to increasing length of incubation time was clearly evident. The LD_{50} for 9-day embryonated eggs was 1/115 while that for 15-day eggs was 1/13 of a guinea pig M.L.D. This difference must be taken into account if embryonated eggs are to be used for diphtheria toxin or antitoxin standardization.

Inoculation of the chorio-allantois of 14-day embryonated eggs with a heavy dose of recently isolated cultures of *C. diphtheriae* from which preformed toxin was removed by repeated washing provoked local pseudomembrane-like lesions that were similar in nearly every respect to those encountered in the natural disease.

Seventy to 75 per cent of eggs infected in this manner died from the effect of toxin produced by the bacteria that grew in the outer layers of the pseudomembrane-like lesion. No invasion of the blood stream or localization by the bacteria within the embryo took place.

Survival of 25 to 30 per cent of the embryos experimentally infected appeared to represent a final balance between the growth rate of the bacterial population on the one hand and the migration to and activities of leukocytes at the site of inoculation on the other. Successive reciprocal waves of bacterial growth and leukocytic migration succeeded one another at regular intervals. In certain instances the accumulation of inflammatory cellular debris appeared to promote conditions unfavorable to the bacterial population. Under these circumstances, the *C. diphtheriae* may be completely eliminated or in some way their capacity to produce toxin is inhibited or the diffusion of toxin through the inflammatory layer does not take place.

Some of the implications suggested by this experimental approach to problems of pathogenesis and, in a restricted sense, to the epidemiology of diphtheria are discussed.

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LEGENDS FOR FIGURES

FIG. 1. Gross appearance of the pseudomembrane-like reaction on the chorio-allantoic (C-A) membrane 18 hours after inoculation. Approximately × 2.

FIG. 2. Gross appearance of the pseudomembrane-like reaction of the C-A membrane 48 hours after inoculation. There is considerable thickening and dessication of the lesion. Approximately × 2.

FIG. 3. The C-A membrane 3 hours after inoculation. A few polymorphonuclear cells are accumulating on the ectodermal surface, and many of them are uniformly scattered throughout the mesoderm. Wright's stain. × 93.

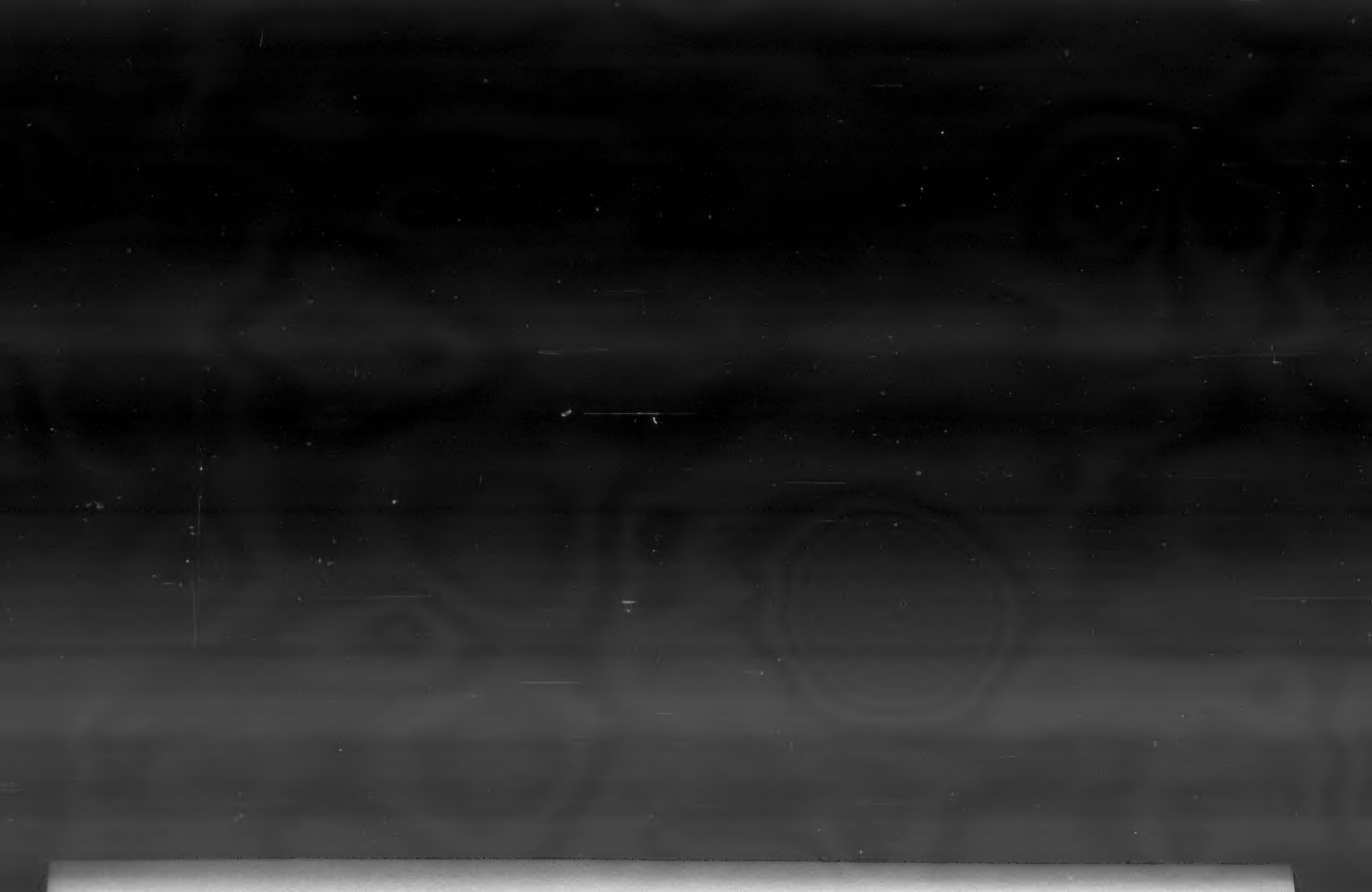
FIG. 4. Smear of exudate from the 3-hour reaction on the C-A membrane shown in Figure 3. Corynebacteria in typical clumps are present in great numbers. Polymorphonuclear cells are at a minimum. Methylene blue stain. × 1000.

FIG. 5. The exudate on the C-A membranal surface at 6 hours. The ectodermal surface at this point is overlaid by an accumulation of polymorphonuclear cells. The ectodermal epithelial cells are degenerating or necrotic. Wright's stain. × 250.

FIG. 6. Smear of the exudate on the C-A membrane in Figure 5. Most of the bacilli have been phagocytized. Methylene blue stain. × 1000.

FIG. 7. C-A membrane 24 hours after inoculation. There are several strata of coagulated and hyaline inflammatory cellular debris. The intensely staining layer of bacteria is clearly visible. Ectodermal epithelium is everywhere intact. The mesoderm contains a minimum of inflammatory reaction. Wright's stain. × 110.

FIG. 8. Smear of exudate from the surface of the C-A membrane in Figure 7. The bacilli in clumps appear to be actively multiplying. Methylene blue stain. × 1000.



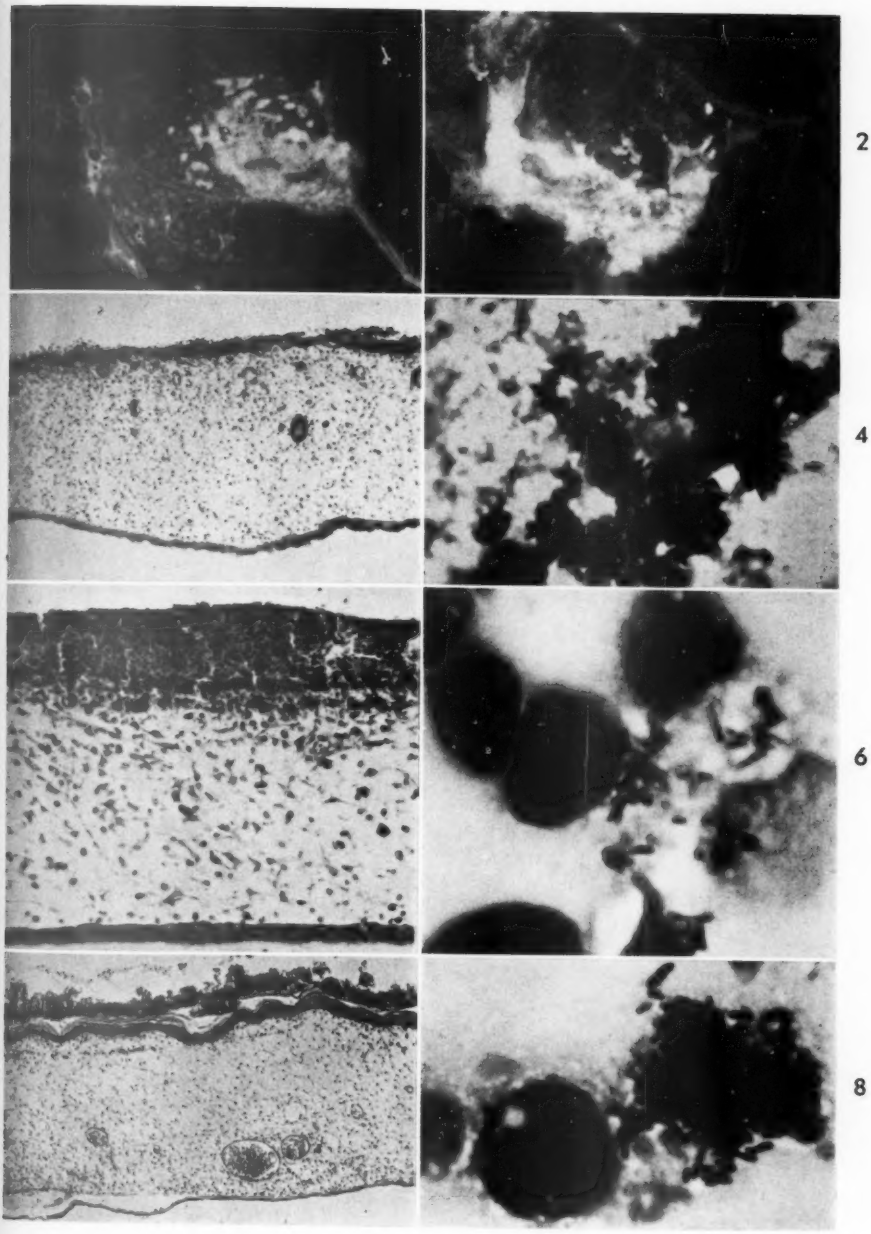

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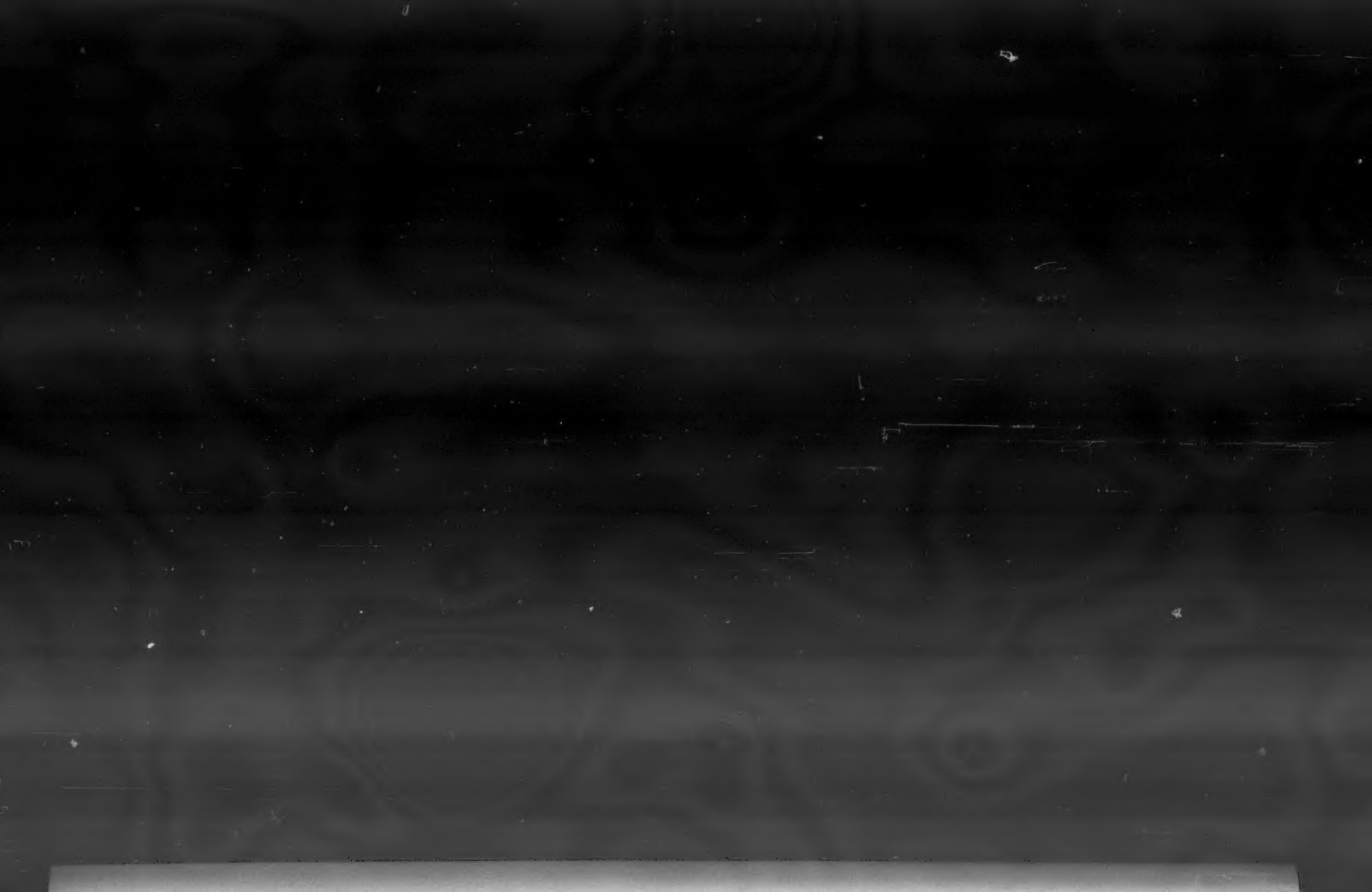
FIG. 9. Gross appearance of the C-A membranal reaction 72 hours after inoculation. The pseudomembranelike lesion is quite thick and leathery and has a typical dead white glistening appearance. Numerous small hemorrhages are scattered throughout. Approximately × 2.

FIG. 10. C-A membrane 72 hours after inoculation. There is complete destruction of the ectodermal epithelium. The surface accumulation of inflammatory cells contains scattered deeply staining masses of bacteria. The injury to the mesodermal blood vessels in the form of thrombosis, hemorrhage and margination of thrombocytes is indicative of the effect of diphtheria toxin. Wright's stain. × 45.



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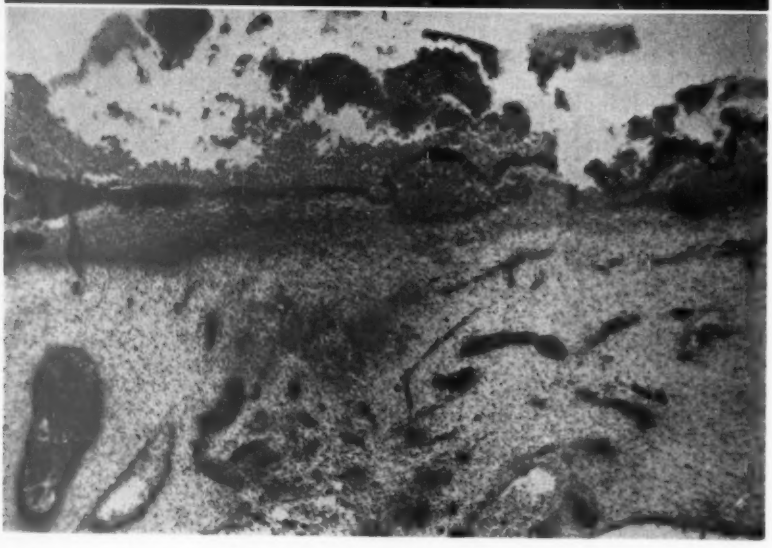

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HYALURONIDASE-SENSITIVE ACID MUCOPOLYSACCHARIDES IN LIPOSARCOMAS

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The purpose of this article is to report the results of staining liposarcomas for acid mucopolysaccharides (AMP). It was found that many liposarcomas with different histologic appearances contained abundant AMP material which was sensitive to the action of bovine testicular hyaluronidase. The AMP material was found both intra- as well as extracellularly in liposarcomas and in the primitive fat organs of a fetus. The observations suggest that fat cells have the ability to produce acid mucopolysaccharides.

Material and Methods

Tissue sections from 30 examples of liposarcoma and from some fetal tissues were examined microscopically. The tissues had been fixed for varying periods of time, usually in neutral formalin. The lesions were categorized according to various microscopic features, and the diagnosis of liposarcoma was established on the basis of existing morphologic criteria. Paraffin sections were stained in all cases by hematoxylin and eosin and by the Mowry[1] improved procedure for staining acid mucopolysaccharides. This method is a modification of that developed by Hale[2] and depends upon the ability of AMP to combine with colloidal iron, following which the location of the iron is demonstrated by the Prussian blue reaction. In addition, an Alcian blue[3] technique was used in some cases and produced similar but less striking results. Other stains, such as toluidine blue, Mayer's mucicarmine, oil red O, Snook's reticulum[4]* and Masson's trichrome[5]* were also used in several instances. The periodic acid-Schiff (PAS) reaction, with and without diastase digestion, was tested in some of the sections.

In conjunction with the stains for AMP, parallel sections were pretreated with bovine testicular hyaluronidase for one hour at room temperature (Wydase; Wyeth Laboratories, Philadelphia, Pennsylvania). For use, 150 turbidity reducing (TR) units were dissolved in 1 cc. of physiologic saline. Tissue sections were covered with this solution. Sections of fetal umbilical cord were used as controls and showed practically complete disappearance of the positive staining for AMP when pretreated with hyaluronidase. Also, sections of a synovial cyst containing fluid reacted in a similar fashion. In contrast, the epithelial mucin in carcinomas and in normal tissues, although staining strongly with the AMP stain, showed no sensitivity to hyaluronidase.

Results

Most of the liposarcomas gave a strong blue-staining reaction with the AMP stain (Table I). This reaction was completely or almost completely prevented by pretreatment with hyaluronidase (Figs. 1 and 2).

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* Modified at the Armed Forces Institute of Pathology.

The reaction was in most cases so marked that it could easily be seen by naked eye examination of the slides. Microscopically, the AMP appeared granular and was located largely in extracellular spaces but also was present within the vacuolated cytoplasm of lipoblasts (Fig. 3). In several cases a combined Alcian blue and oil red O staining procedure performed on frozen sections revealed the presence of both AMP and fat within the same lipoblast. The AMP substance was slightly eosinophilic and stained pink with Mayer's mucicarmine. In some cases attempts were made to demonstrate glycogen by use of the PAS stain, with

TABLE I

HYALURONIDASE-SENSITIVE AMP IN LIPOSARCOMAS

Histologic type *	Number	Estimated amount †
Embryonal	13	3 to 4+
Mucoid but not embryonal	9	3 to 4+
Pleomorphic	4	1 to 3+
Spindle cell	1	1+
Differentiated	3	0 to 1+

* The histologic pattern was usually a mixed one. For the purpose of this table, the most prominent or significant microscopic feature has been used to indicate the histologic type. The term "mucoid" is used for those tumors that contain considerable connective tissue mucin.

† 0 = none seen microscopically
1+ = small scattered amounts seen microscopically
2+ = considerable amounts seen microscopically and barely observed by naked eye
3+ = easily seen with naked eye
4+ = very prominent on naked-eye examination

and without pretreatment with diastase, but the results were negative. The Masson trichrome method stained the material reddish to greenish, and intracytoplasmic fat vacuoles were distinctly outlined. With toluidine blue the material was light purple, staining in a definitely metachromatic fashion.

The AMP material was observed in a metastatic liposarcoma as well as in the primary tumor and was present in tumor emboli. The Snook reticulum stain demonstrated the substance to be argyrophilic; both granular and fine wavy fibrils were stained. The fibril formation was closely associated with lipoblasts, some fibrils appearing to be within or on the surface of these cells (Fig. 4).

The amount of AMP in various histologic types of liposarcomas was compared. In most instances the moderately well differentiated and pleomorphic as well as the myxoid or embryonal forms exhibited prominent AMP content, and hyaluronidase sensitivity was easily demonstrated. In the more embryonal and myxoid forms, however, there was relatively more hyaluronidase-sensitive AMP than in tumors composed

of obviously vacuolated though neoplastic fat cells. In the well differentiated liposarcomas in which the fat cells were similar to normal adult fat cells, the amount of AMP was usually slight or negligible, and in areas of dense fibrosis, the substance was usually insignificant. This was not necessarily true, however, where the neoplastic lipoblasts assumed a spindle shape. From a morphologic point of view, the impression was gained that collagen formation trapped spindle cells, which then became relatively inactive and assumed the histologic appearance of fibroblasts.

In some of the liposarcomas there were foci of chondrogenesis. These regions stained deep blue with the colloidal iron stain, but were only slightly sensitive to hyaluronidase. Fetal cartilage was found to react in a similar way to the colloidal iron stain and hyaluronidase.

In order to ascertain whether fetal fat also contained a similarly reacting AMP substance, a human fetus (23 cm., 380 gm., approximately 15 weeks' gestation) was serially sectioned. The fat islands in the subcutaneous tissue and in other areas were found to contain considerable amounts of hyaluronidase-sensitive AMP (Fig. 5). In another fetus of approximately 12 weeks' gestation, the primitive fat organs of the subcutaneous tissue were not developed to the stage at which they could be recognized. Normal adult adipose tissue was found to contain little if any demonstrable AMP.

A preliminary investigation of other tumors revealed that 8 examples of rhabdomyosarcoma contained little if any demonstrable hyaluronidase-sensitive AMP substance. Five of these were of the embryonal type. The developing striated muscle in the previously mentioned fetuses contained no demonstrable hyaluronidase-sensitive AMP.

Discussion

From this study it is apparent that many liposarcomas contain relatively large amounts of an acid mucopolysaccharide giving a histochemical reaction characteristic of hyaluronic acid or chondroitin sulfate. Although there are many publications on the subject of acid mucopolysaccharides and some investigators have studied various tumors for the presence of such material,[6-9] there is scarcely any reference to lesions of adipose tissue in this regard. Karl Meyer,[10] in his table of the distribution of acid mucopolysaccharides, lists a liposarcoma in his group I which supposedly contains only hyaluronic acid as the principal acid mucopolysaccharide.

Our studies show that in most liposarcomas nearly all the acid mucopolysaccharide present is completely altered by bovine testicular hyaluronidase in such a way that it no longer stains with AMP stains. In

foci of cartilaginous metaplasia, however, the acid mucopolysaccharides were found to be only slightly sensitive to testicular hyaluronidase. With the method used, fetal cartilage reacted in a manner similar to that of the chondromatous foci. Meyer and Rapport [11] have found hyaline cartilage to contain chondroitin sulfate A and C, and both of these acid mucopolysaccharides have been found to be susceptible *in vitro* to the enzyme of bovine testicular hyaluronidase. Pearse,[12] however, has observed that tissue sections of cartilage do not react to testicular hyaluronidase as might be expected on the basis of *in vitro* tests. It is probable that by altering the conditions of the hyaluronidase treatment and by using fresh tissue, different results could be obtained.

The production of the acid mucopolysaccharide content in connective tissue ground substance has been attributed to the fibroblast by a number of investigators.[13–17] In some instances the authors have not excluded the possibility that associated adipose tissue cells might also have been concerned in the production of the AMP. The microscopic demonstration of large amounts of hyaluronidase-sensitive AMP within the cytoplasm of fetal or neoplastic fat cells offers evidence that these elements under certain metabolic conditions can elaborate this substance.

Further support for production of hyaluronidase-sensitive AMP by lipoblasts is given in an example of metastasizing liposarcoma. In this instance a primary tumor of the thigh metastasized to the lung, adrenal, and kidney. In the lung at necropsy, there were discrete metastatic tumor deposits. Microscopic examination revealed considerable intra- and extracellular hyaluronidase-sensitive AMP substance in these lesions. In addition, many of the pulmonary blood vessels contained tumor emboli in which the same AMP material was demonstrated.

The results of this study, therefore, indicate that hyaluronidase-sensitive AMP may be formed by neoplastic and immature fat cells. This observation seems of particular interest in view of Siperstein's recent review of the interrelationships between glucose and lipid metabolism.[18] According to this author, there is now accumulated biochemical evidence that the major site of conversion of carbohydrate to fat is in the adipose tissue of the body.

Summary

1. Thirty examples of liposarcoma were investigated for the presence of hyaluronidase-sensitive acid mucopolysaccharide (AMP) material.

2. Most of these lesions contained abundant hyaluronidase-sensitive acid mucopolysaccharides. The embryonal and mucoid neoplasms contained relatively more than the other forms. Little, if any, AMP was present in very well differentiated nonmucoid tumors.

3. Fetal adipose tissue was also found to contain a large amount of hyaluronidase-sensitive AMP, but scarcely any was found in normal adult fat.

4. The existence of hyaluronidase-sensitive AMP material in embryonal adipose tissue and in liposarcomas suggests that fat cells have the capacity to contribute to the formation of connective tissue ground substance.

Addendum

Since this article went to press, additional studies have revealed that in so-called serous atrophy of fat the adipose tissue contains less fat and a conspicuous amount of hyaluronidase-sensitive acid mucopolysaccharides. This observation offers additional support to the concept that under certain metabolic conditions the fat cell is capable of producing hyaluronidase-sensitive acid mucopolysaccharides.

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LEGENDS FOR FIGURES

FIG. 1. Myxoid liposarcoma (AFIP Acc. No. 928926; AFIP Neg. No. 59-5653, 59-5655, and 59-5654). (A) A primitive embryonal liposarcoma in which the cells are poorly differentiated and separated by a prominent myxoid stroma. Hematoxylin and eosin stain. × 400.

(B) Section of the same tumor stained for acid mucopolysaccharides. Colloidal iron stain. × 6½.

(C) Similar to section (B) but pretreated with bovine testicular hyaluronidase before staining for AMP. × 6½.

FIG. 2. Pleomorphic liposarcoma (AFIP Acc. No. 911753; AFIP Neg. No. 59-5146; 59-5148, 59-5147). (A) The tumor cells show variation in size and shape, and many contain cytoplasmic fat vacuoles. Hematoxylin and eosin stain. × 600.

(B) Section of the same tumor stained for acid mucopolysaccharides. Colloidal iron stain. × 4½.

(C) Similar to section (B) but pretreated with bovine testicular hyaluronidase before staining for AMP. × 4½.

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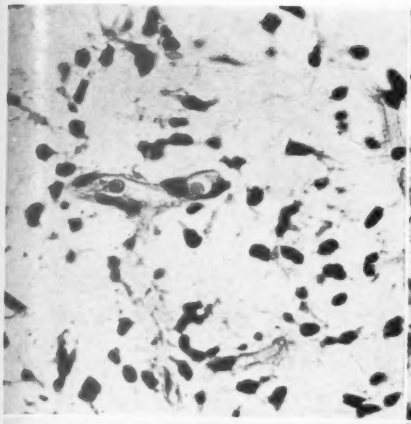
1C

1A



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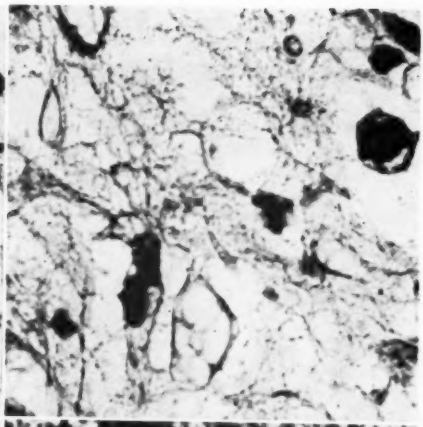

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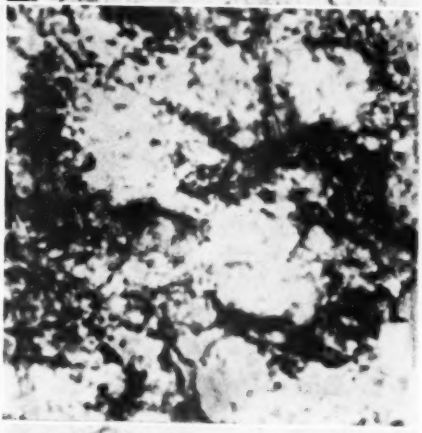

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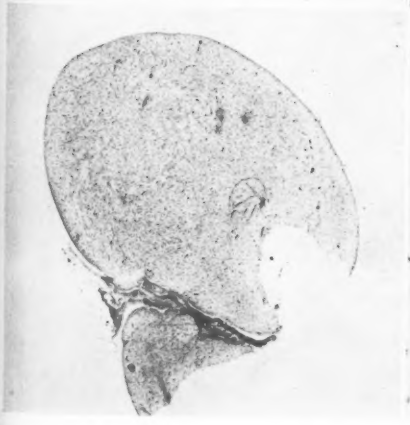

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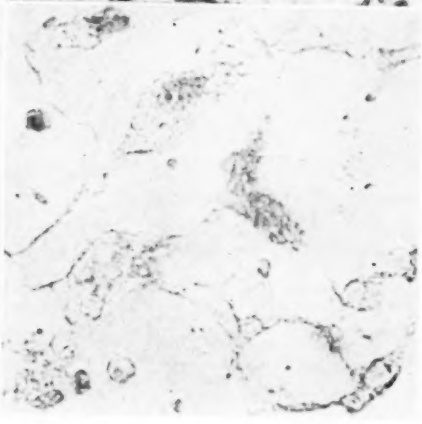
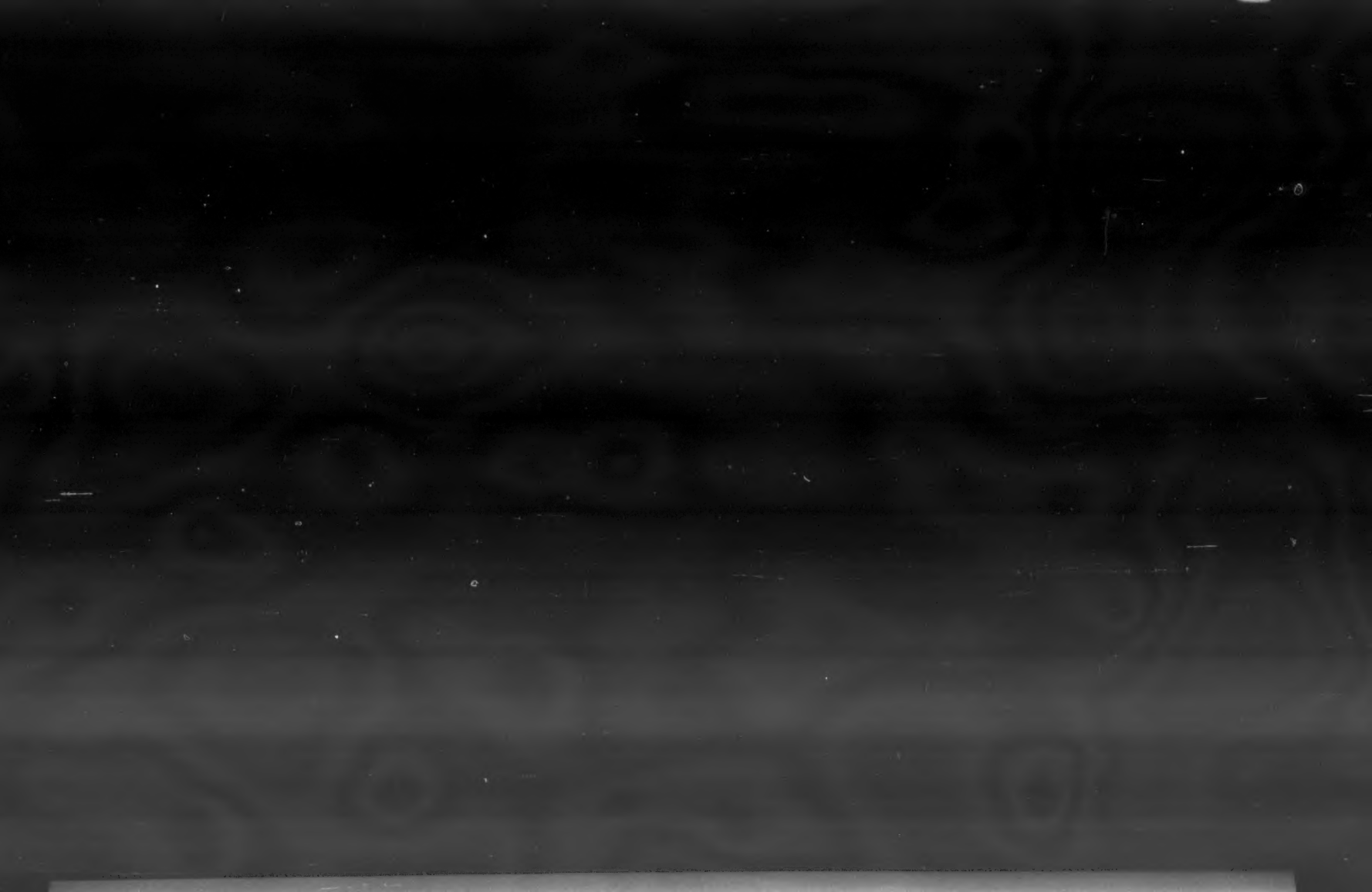
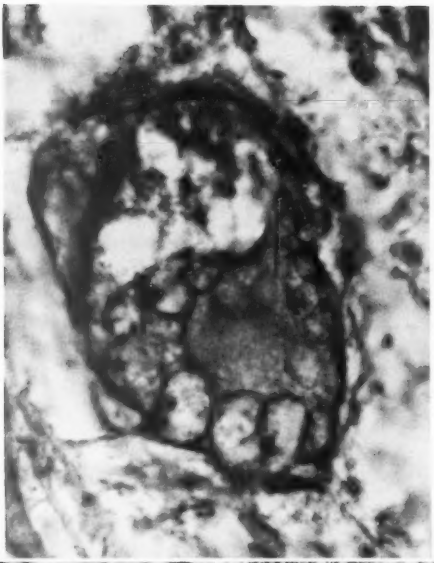


FIG. 3. A lipoblast with intra- and extracellular AMP-positive substance (AFIP Acc. No. 814230; AFIP Neg. No. 59-5959). Colloidal iron stain. × 1400.

FIG. 4. A metastatic liposarcoma in the lung, showing granular and fibrillar argyrophilic substance (AFIP Acc. No. 873043; AFIP Neg. No. 59-5961). Snook's reticulum stain. × 1400.

FIG. 5. Fetal fat showing the presence of AMP material especially concentrated in the primitive fat organs (AFIP Acc. No. 902681; AFIP Neg. No. 59-5965). Colloidal iron stain. × 80.





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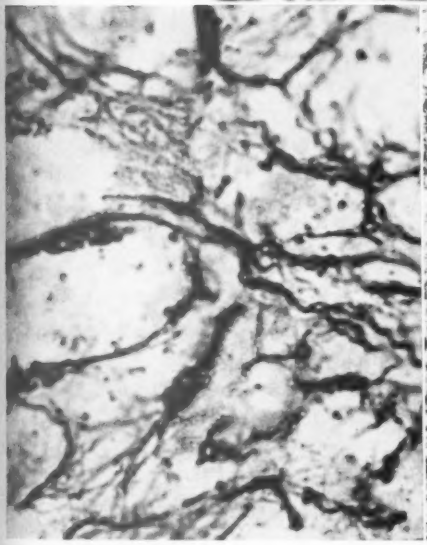

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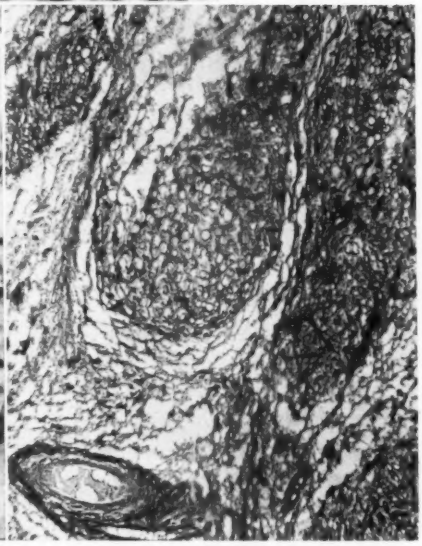

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